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Although the cause of breast cancer has not been identified yet, there is enough clinical and experimental evidence that full term pregnancy reduces the lifetime risk of developing breast cancer, a protective effect that can be mimicked by treatment of virgin animals with the placental hormone chorionic gonadotropin (hCG). *In vivo* this hormone inhibits both the initiation and progression of rat mammary carcinomas, and *in vitro* the proliferation of human breast epithelial cells (HBEC). Work performed under this grant application has led the PI to determine that treatment of immortalized, chemically transformed and malignant HBEC with hCG activates programmed cell death genes even before an arrest of cell growth has becomes evident. It also acts as an inhibitor of cell proliferation, utilizing different pathways for either activating programmed cell death genes or inhibiting specific cell cycle dependent kinases, depending upon the degree of expression of neoplastic phenotypes. The relevance of these findings lies in the potential use of hCG as a chemopreventive and chemotherapeutic agent in breast cancer, utilizing the detection of activation of programmed cell death genes as an early end point in the action of this hormone on the target tissues.

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Hormonal Control of Breast Cancer Cell Growth

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INTRODUCTION

Breast cancer is the neoplasm most frequently diagnosed in American women. It is also responsible for 17 percent of female cancer deaths, and the number one cause of cancer-related death in non-smoking women (1,2). These dismal statistics are aggravated by the facts that the incidence of this disease is steadily rising in most western societies and in oriental countries traditionally known to have a low incidence of breast cancer (3-5), and by the lack of a clear understanding of the cause of this worldwide increase. Breast cancer has long been recognized to be a hormone dependent malignancy (6,7). Combined clinical and experimental studies have determined that both hormone deprivation and hormone supplementation have a preventive and therapeutic value (7). Other important endocrinological influences have been found to modify the risk of developing breast cancer. Increased risk is associated with early menarche, late menopause, nulliparity or late first full term pregnancy, while opposite conditions, such as late menarche, early pregnancy and early menopause exert a protective effect (8-11). Although the cause and the time of initiation of the carcinogenic process are not known, epidemiological, clinical, and experimental data have identified the period between menarche and the first full term pregnancy as a "window" of critical importance in the lifetime risk of developing breast cancer. The understanding of the complex interactions involved in the initiation and progression of breast cancer requires the design of studies in which all possible variables are carefully controlled. *In vivo* and *in vitro* experimental models provide the ideal conditions for exploring the mechanisms through which these interactions influence the risk of mammary cancer development (12-16).

The widely utilized animal model of induction of rat mammary tumors by administration of the chemical carcinogen 7,12-dimethylbenz(a)anthracene (DMBA) to Sprague-Dawley rats (8,12-14) has allowed the PI to determine that the differentiation of the mammary gland is the single most important factor in determining the susceptibility or resistance of the mammary gland to develop malignancies. Full differentiation of the mammary gland occurs after a full term pregnancy, although treatment of young virgin rats with the placental hormone human chorionic gonadotropin (hCG) exerts an effect on the mammary gland's lobular development, inhibition of cell proliferation, increase in the DNA repair capabilities of the mammary epithelium and activation of genes controlling programmed cell death similar to that induced by pregnancy (17-22). Both processes inhibit the initiation of chemically induced mammary carcinogenesis, and hCG also inhibits the progression of rat mammary carcinomas, and the proliferation of immortalized, chemically-transformed and malignant human breast epithelial cells *in vitro* (17-29).

Chorionic gonadotropin is a glycoprotein hormone secreted early in pregnancy by the developing embryo (30). Its main known function, which is virtually identical to that of the pituitary luteinizing hormone (LH), is the stimulation of the production of gonadal steroid hormones through its interaction with the lutropin-choriogonadotropin-receptor (LH-CG-R) (31). This receptor has been traditionally known to be present in the granulosa cells of the ovary in the female and in the testicular Leydig cells in the male. In the ovary, upon interaction with its receptor, hCG increases adenylyl cyclase activity, an effect mediated by intracellular membrane associated G proteins; this, in turn, results in cAMP increases, leading to steroid and polypeptide

hormone synthesis (30-32). An understanding of the mechanisms through which hCG inhibits the proliferation of both immortalized and neoplastic human breast epithelial cells would provide researchers indispensable clues on the role of this important pregnancy hormone in the prevention of breast cancer. The fact that hCG inhibits the growth of human breast epithelial cells (HBEC) treated *in vitro* indicates that this hormone has a direct effect on cell growth, observation that led us to postulate that hCG acts via a receptor. We proposed to elucidate whether the interaction of this hormone with its putative receptor might activate genes controlling programmed cell death (PCD), or other genes that might lead to the arrest of the cells in specific phases of the cell cycle (34-36).

Subject

The fact that hCG has a direct inhibitory effect on the proliferation of human breast epithelial cells *in vitro* (27-29) led us to postulate that one of the mechanisms responsible of this inhibition is the activation of genes controlling programmed cell death (PCD), leading to apoptosis, as it has been shown in other systems (34-36). PCD and apoptosis are physiological forms of active cell death (34-39). Both processes have been associated with specific phases of development that control cell proliferation and differentiation. In addition, both hormone withdrawal and the administration of certain chemotherapeutic agents have been shown to induce apoptosis and to inhibit mammary tumor development *in vivo* and cell proliferation *in vitro* (40,41). PCD is regulated through a phylogenetically conserved mechanism, that in C. elegans has been shown to be regulated by the ced-3, ced-4 and ced-9 genes (42,43). The gene product of ced-3 has homology to a cysteine protease, the mammalian interleukin-1- converting enzyme (ICE). Subsequent studies have revealed the presence of several ICE-related genes, including CPP32/Yama, or apopain (44-46). *In vitro* studies have suggested that a protease cascade involving the ICE protease family acts to transduce apoptotic signals, such as TX/ICH2/ICE rel-II (47,48), which can cleave the precursor of CPP32/Yama (46). The demonstration that the inhibition or mutation of these genes inhibit apoptosis induced by various stimuli have confirmed their important role in driving apoptosis (44,49-51).

Progression of apoptosis in mammalian cells has also been associated with increased expression of testosterone repressed prostate message-2 (TRPM2) gene (52-54). Enhanced expression of TRPM2 and of transforming growth factor (TGF)- has been demonstrated in human breast cancer cells following estrogen ablation (55). The product of the proto-oncogene bcl2 is also known to play a role in promoting cell survival and inhibiting apoptosis (56-59). However, the existence of bcl-2 related gene products that accelerate programmed cell death has been reported (60,61). One of these gene products, termed bax, has been shown to homodimerize and to form heterodimers with bcl-2; it has been suggested that the ratio of bcl2 to bax determines survival or death following an apoptotic stimulus (62). C-myc has been demonstrated to play a crucial role in the induction of apoptosis in several cell types, either through p53 dependent or p53-independent pathways (63,64). An important downstream target for p53 is the WAF1/ClP1 gene, which is transcriptionally regulated by wild type p53 (65). P2l, the gene product of WAF1/ClP1, acts as a potent inhibitor of cyclin dependent kinases (66,67) and thus may mediate the p53 induced cell cycle arrest and apoptosis following DNA damage (68).

The maintenance of the homeostatic balance between cell proliferation, differentiation, and cell death in human cells requires intrinsic regulatory mechanisms that counterbalance growth stimulatory effects. It is known that checkpoint controls are modulated by the synthesis and degradation of cyclins and phosphorylation by cyclin-dependent kinases (cdks) (69-72). Negative regulatory forces are provided by tumor suppressor proteins such as the p53 and the retinoblastoma (pRb) proteins (73) and by cyclin-dependent kinase inhibitors, such as roscovitine, that inhibits the kinase activity of cdc2/cyclin B, cdk2/cyclin A, and cdk2/cyclin E complexes (69,74-79).

Purpose

The present work was designed with the purpose of clarifying the mechanisms through which hCG affects the *in vitro* growth of HBEC. For these purposes, gene expression was studied in hCG-treated normal immortalized human breast epithelial cells MCF10F (80,81); in benzo(a)pyrene (BP)-transformed BPlE and BPlE-Tp cells, derived from MCF-10F cells (82,83), and the malignant breast and urothelial cancer cells MCF-7 and T24, respectively (82,83). The effect of hCG was compared with that of roscovitine, which is known to prevent the cell cycle progression of mammalian cells at the G1/S and the and G2/M checkpoints and blocks the transactivation of cyclin A by myc (76-79). Comparisons were also made with the effect of resveratrol, a trihydroxystilbene present in red wines, grapes and berries (84,85), which has been shown to exert a cancer preventive effect (85). Information obtained in these systems was correlated with our studies on the effect of hCG treatment on cell cycle progression, determined by flow cytometry, and on the expression of genes that modulate PCD.

Scope of Research

Our studies, that were initiated with the purpose of clarifying the mechanisms through which pregnancy reduces the incidence of mammary cancer (2-12), led us to discover that a single placental hormone, human chorionic gonadotropin (hCG) administered to virgin rats inhibits both the initiation and progression of chemically induced mammary cancer (18-20,86,87). This protective effect was the result of the induction of mammary gland differentiation, manifested as profuse lobular formation with elimination of undifferentiated structures, mainly terminal end buds (TEBs), reduction of the proliferative activity of the mammary epithelium (8) and increased synthesis of inhibin, a growth factor with tumor suppressor activity (22). HCG treatment inhibits tumor initiation when given before the carcinogen, and tumor progression when administered even 20 days after the carcinogen (18). This phenomenon is mediated by the induction of an increase in the expression of TRPM2, ICE, bcl-XS, c-myc and p53, and elevation in the apoptotic index in the non-tumoral mammary glands (23,24). HCG treated animals develop considerably fewer tumors than controls, and their mammary adenocarcinomas exhibit an increased expression of p53, c-myc and ICE genes in comparison with the levels detected in the adenocarcinomas developed by the animals treated with DMBA alone. These observations indicate that hCG induces programmed cell death in the mammary gland initiated in the carcinogenic process, that this process is p53 dependent, and is modulated by c-myc expression. Our data also indicate the possibility that a cell death program dependent of the bcl2 family exists, because of the potential involvement of p53, bcl-XS and Bax in apoptosis (24-26). The objective of this proposal was to validate

observations made in the experimental animal model for their extrapolation to the human disease. This approach was supported by our observations that hCG inhibits the proliferation of human breast epithelial cells *in vitro.* The inhibition of *in vitro* cell growth of both the spontaneously immortalized human breast epithelial cell line MCF-10F and of the breast adenocarcinoma metastatic cell line MCF-7 indicate that this hormonal treatment is of great potential value for the treatment of already established breast cancers, in addition of representing a promissory model for the design of protocols of breast cancer prevention.

Background of Previous Work

Influence of reproductive factors and breast cancer risk: It has long been known that the incidence of breast cancer is greater in nulliparous than in parous women (9-11). Changes in lifestyle, that in turn influence the endocrinology of women, have been observed during the last decades in American women, namely a progressive decrease in the age of menarche and a progressive increase in the age at which a woman bears her first child (9,86,87). The significance of these changes is highlighted by the reduction in breast cancer risk associated with late menarche and the completion of a full term pregnancy before age 24, with further reduction in the lifetime breast cancer risk as the number of pregnancies increases (11,88). The fact that women from different countries and ethnic groups exhibit a similar degree of parity-induced protection from breast cancer, regardless of the endogenous incidence of this malignancy (88), suggests that the reduction in breast cancer risk associated with early first full-term pregnancy does not result from extrinsic factors specific to a particular environmental, genetic, or socioeconomic setting, but rather from an intrinsic effect of parity on the biology of the breast (which nevertheless may be modified by environmental, genetic, or other factors) (1-4,9-11,86-88). These observations imply that an early first full-term pregnancy results in a change in the breast that either directly or indirectly decreases the lifetime risk of breast cancer development. It has been hypothesized that this protection is the result of the terminal differentiation of the mammary gland that reduces the susceptibility of the mammary epithelium to carcinogenesis (89, 95). These observations indicate that the terminally differentiated state of lactation should be reached for attaining protection.

Our studies indicate that the first pregnancy modifies dramatically the architecture of the breast ((94,95). The development of this organ is a progressive process of growth initiated at childhood that evolves through a progressive elongation and branching of ducts. At puberty starts the sprouting of lobular structures, which evolve from the undifferentiated lobule type 1 (Lob1) to the more differentiated Lob2 and Lob3, that originate under the cyclic hormonal stimulation of the ovaries (96,97). Those Lob1 present in the breast of young nulliparous women have a high rate of cell proliferation and a high content of estrogen (ER) and progesterone (PR) receptors, while the more differentiated Lob 2, Lob 3, and the fully differentiated secretory Lob 4 that develops during pregnancy show a progressive decrease in both cell proliferation and steroid hormone receptor content (96,97). These more differentiated lobules express, instead, specific markers associated with cell differentiation, such as inhibin and mammary derived growth inhibitor (MDGI) (98).

Role of pregnancy and chorionic gonadotropin in mammary cancer inhibition: The direct association of breast cancer risk with the prolongation in the period encompassed between menarche and the first full term pregnancy, as well as the protection afforded by pregnancy have been partially explained by experimental studies performed in our laboratory (89,90,92,93). We have demonstrated that mammary cancer in rodents can be induced with the chemical carcinogen DMBA only in the young nulliparous females. Completion of pregnancy prior to carcinogen exposure, on the other hand, prevents carcinoma development (89,90,99). This preventive effect has been attributed to the induction of differentiation of the mammary gland, which is in great part mediated by chorionic gonadotropin (CG), a glycoprotein hormone first secreted by the fertilized egg and later on by the placenta (18,86,99). Its detection in the maternal circulation is the only established way of ascertaining the presence of pregnancy (100).

We have demonstrated that the protective effect of pregnancy is mediated by hCG, since virgin rats treated for 21 days with a daily intraperitoneal injection of hCG exhibit a dose-related reduction in tumor incidence and number of tumors per animal, with maximal effect observed with a 100 IU treatment (18,86,99). This phenomenon is in great part mediated by the induction of mammary gland differentiation, inhibition of cell proliferation, increase in the DNA repair capabilities of the mammary epithelium, decrease binding of the carcinogen to the DNA, and activation of genes controlling PCD (18,34-36,86,99,101-103).

Role of hCG in breast cancer progression: Our studies on the effect of hCG on the differentiation of the mammary gland led us to postulate the possibility that hCG might be useful for the prevention of cancer development in women. A major drawback for pursuing this goal was posed by lack of knowledge of when breast cancer initiates in women, which makes it impossible to determine when to institute a truly "preventative" hormonal treatment. Thus, It had to be assumed that all women are at risk of being the carriers of "initiated" lesions, and any type of treatment has to be proven to inhibit the progression of those putatively initiated cells, or at least not to cause tumor progression. Based upon our previous observations that the chemical carcinogen DMBA induces neoplastic transformation in the mammary gland by acting on the highly proliferating TEBs of the virgin animal (89,90,104), and that once initiated these structures progress to intraductal proliferations (IDPs) within 3 weeks of exposure to the carcinogen (8,90,104), we tested the effect of hCG on tumor progression by administering 8 mg DMBA /100 g body weight to 45 day-old virgin Sprague-Dawley rats. Twenty days later, when IDPs were already evident, the animals were treated with 100IU/hCG per day for 40 days (DMBA+hCG group). Age matched untreated, hCG-, and DMBA +saline treated rats were used as controls. Tissues were collected at the time of DMBA administration and at 5, 10, 20, and 40 days of hCG injection, and 20 days post-cessation of treatment. This treatment inhibited the progression of mammary carcinomas by stopping the progression of early lesions, i.e., IDPs and carcinomas in situ (CIS), findings that indicated that hCG has a significant potential as a chemopreventive agent not only before the cell is initiated, but after the carcinogenic process has been initiated and is vigorously progressing. Ours was the first report to indicate that a hormone preventive agent like hCG is able to stop the initiated cells by inhibiting the formation of the intermediate step represented by the CIS, that ultimately results in a lower incidence of invasive tumors (101,105,106).

Effect of hCG on programmed cell death gene expression: Treatment of virgin rats with the placental hormone hCG induces differentiation of the mammary gland, depresses cell proliferation, and increases DNA repair of DMBA-damaged cells, and induces the synthesis of inhibin, a gonadal protein with tumor suppressor activity (18,86,90,99). Treatment initiated after the administration of the chemical carcinogen DMBA, as described above, inhibits the progression of mammary carcinomas. This inhibition has been shown to be associated with the activation of genes that are known to be responsible of programmed cell death and apoptosis (34-36,107). Northern blot analysis of mammary gland polyadenylated RNA using gene specific probes for ICE, TRPM2, p53, c-myc, and bcl-XS revealed that there was a remarkable induction of these apoptotic genes in the mammary glands of rats treated with hCG, either alone or after DMBA, whereas very little or no significant changes were found in the mammary glands of rats treated with DMBA (101). The expression of bcl2, bcl-XL, TGF-α and TGF-β, on the other hand, was not significantly affected by either hCG or DMBA treatments. The effect of hCG on the activation of programmed cell death genes was specific for the mammary glands, since the hormonal treatment did not modify their expression in the ovary, even though this is the target organ of hCG action (101). The activation of programmed cell death genes in the mammary gland of hCG treated rats occurred as early as five days after the initiation of the hormonal treatment; it was maintained throughout the treatment period, and some genes remained activated up to 20 days post-cessation of treatment. Gene activation was followed by fragmentation of intranucleosomal DNA, detected as the formation of apoptotic bodies, or apoptotic index, in the mammary epithelium of lobules and in the few tumors that developed. We concluded that hCG treatment of virgin rats in which the carcinogenic process had been initiated in the mammary gland with the chemical carcinogen DMBA, in addition to inducing differentiation of the mammary gland it induced programmed cell death, an effect we postulated is p53 dependent, and modulated by c-myc expression. Our results suggest the possibility of the existence of a cell death program that is dependent on the bcl2 family because of the potential involvement of p53, bcl-XS and Bax in apoptosis (101-103). It was concluded that the use of agents like hCG, that induce apoptosis, may constitute a useful approach for the prevention and therapy of breast cancer.

***Effect of hCG on inhibin synthesis and early response gene expression*:** Our observations that the hCG-induced differentiation of the mammary gland is associated with the synthesis of inhibin, a heterodimeric protein that is structurally related to the transforming growth factor-β (TGF-β) family (108-110), led us to test whether inhibin was also involved in the regression of DMBA-induced rat mammary carcinomas. For these purposes, virgin rats that received 8 mg DMBA /100 g body weight when they were45 days old; and 20 days later they were injected daily with 100IU/hCG for 40 days, as described above, with age-matched untreated, hCG-, and DMBA +saline treated rats used as controls, were utilized for the collection of mammary and tissues and ovaries at the time of DMBA administration and at 5, 10, 20, and 40 days of hCG injection and 20 days post-cessation of treatment. Total and polyadenylated RNAs were probed for inhibin A, B, c-myc, c-fos, and c-jun. The mammary glands of hCG-treated animals exhibited elevated expression of Inhibin A (1.5 to 4.0 fold) and Inhibin B (1.5 to 3.0 fold), from the 5th day of hCG treatment up to 20 days post-treatment. The expression of these genes was also enhanced by hCG in the DMBA treated group, whereas no changes occurred in the animals treated with DMBA alone. The hormonal treatment markedly increased the expression of c-myc and c-jun by 4-7 fold and 2-3 fold, respectively. No significant changes were found in the levels

of c-fos expression, and DMBA treatment alone did not modify the expression of these genes. Immunohistochemical staining showed a very strong immunoreactivity for inhibin and subunits that became evident in the lobular epithelium by the 10th day of treatment and reached a peak of expression at the 20th day, with a similar pattern of reactivity observed in animals treated with hCG alone or after DMBA. The expression of both subunits remained elevated up to 20 days post-hormone withdrawal, even though the lobular structures had involuted from the well-developed secretory lobules type 3 and 4 to lobules type 2 and 1. The finding that c-myc and c-jun were also elevated at the time of maximal inhibin synthesis indicated that early response genes could be involved in the pathway of hCG-inhibin induced synthesis (111).

Comparative effect of hCG and pregnancy on gene expression. Our findings indicate that under *in vivo* conditions hCG induces a myriad of effects that ultimately result in activation of programmed cell death genes, increased apoptosis, induction of inhibin and milk protein synthesis in the mammary gland leading to the inhibition of cancer development. In order to determine whether specific genes are differentially expressed in the rat mammary gland of animals treated with either r-hCG, the clinically used Profasi (Serono, Norwell, MA), or during pregnancy, in comparison with the virgin rat, and for evaluating whether gene activation was a transitory or long-lasting phenomenon, virgin rats treated for 15 or 21 days with the hormones, were sacrificed after the 15th injection or 40 days after the 21st injection, respectively. Parous animals were either sacrificed at the 15th day of pregnancy or 40 days after weaning of the pups, respectively. Each group was compared with untreated age-matched virgin rats. The permanence of these changes was evaluated by comparison of those changes detected at the 15th day of hormonal treatment or pregnancy with those still present 40 days post-cessation of treatment or breast-feeding respectively. The mammary glands of r-hCG treated animals showed elevated expression of TRPM2 transcripts by 2.5 to 5 folds after the 15-day treatment, and remained elevated thereafter. The rate of elevation was similar to that observed in the pregnant animals. The treatment of virgin rats with r-hCG or Profasi, as well as pregnancy, induced in the mammary glands the expression of the differentiation genes -casein and whey acidic protein, two of the major milk proteins in most species (112-121), which were absent from the mammary glands of untreated virgin controls. The expression of those genes was elevated during both pregnancy and the injection period with the two hormones, and it remained activated up to 40 days post weaning or cessation of treatment respectively. A third cDNA fragment, called Hormone-Induced 1 (HI-1) was expressed following the same pattern observed in -casein and whey acidic protein genes. The sequence homology of HI-1 did not match any previously identified genes, appearing to be a novel gene whose function might be related with process of differentiation. Its role in the protection from carcinogenesis is actively pursued in our laboratory. The fact that casein has a strong antimutagenic activity both *in vivo* in and *in vitro* (112-121) indicates that further identification of the functional role of this protein and of others whose synthesis is stimulated by pregnancy and hCG treatment might provide important clues on the mechanisms through which these hormonal and reproductive influences protect the breast from neoplastic transformation.

BODY

Experimental Methods and Procedures

***Cells*:** The spontaneously immortalized human breast epithelial cells MCF-10F, which originated from the mortal cells MCF-10M, and clones BP1-E and BP1-E-Tp, derived from benzo(a)pyrene (BP) transformed MCF-10F cells, and MCF-7, a human breast carcinoma cell line, all maintained in our laboratory (80-83), and T24, a human bladder carcinoma cell line purchased from the American Tissue Type Collection (Rockville, MD) were used in these experiments. MCF-10F, BP1-E, and BP1-E-Tp cells, were used at passages 130, 60, and 6 respectively. MCF-10F and the BP-transformed cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM)/F-12 (1:1), supplemented with equine serum, insulin, hydrocortisone, epidermal growth factor, cholera toxin, and antibiotics. MCF-7 cells were grown in DMEM/F-12 medium supplemented with 5% fetal calf serum, antibiotics and insulin, and T24 cells in the same medium, but containing 10% fetal calf serum. The cells were cultured at 37^0C in a humidified atmosphere under 5% CO_2.

HCG Treatment and Cell Proliferation: For studying the effect of hCG on the proliferative activity of the cells, confluent cultures were harvested, counted with a hemacytometer and seeded into 96-well plates (Corning Incorporated, New York, NY). In a final volume of 100 ul culture medium per well. Twenty four hours post-plating the cells were treated with 100 IU hCG (Profasi, Serono Laboratories, Inc., Randolph, MA). This dose was selected based on previous experiments that have demonstrated a dose-related effect, with maximal inhibitory response with 100 IU (27-29). Control cells were treated with the same volume of buffer in which the hormone was dissolved. The hormone and the vehicle were replaced daily. Cells were collected at 24 and 120 hours of treatment. Four hours prior to the scheduled time of collection the cells were treated with 10 ul of the cell proliferation reagent WST-1 (Boehringer Mannheim, Indianapolis, IN). Then the optical density of the wells was spectrophotometrically read at 490 nm with an EL-312 microplate reader (BIO-Tek Instruments, Inc., Winooski, VT). Optical density values were plotted against the number of cells counted with the hemacytometer for generating a standard curve. A new standard curve was set up for every experiment. All assay were performed at least twice, and each assay was performed in triplicate wells.

Analysis of Gene Expression: Each one of the cell lines listed above were plated in six T150 flasks (Falcon, Lincoln Park, NJ) concentration of $1x10^4$ cells per cm^2. When the cells reached 80% confluence they were treated with hCG for periods of time ranging from 24 to 120 hours, with daily changes of the hormone-containing medium. Control cells were treated with the same volume of buffer in which hCG was dissolved. The cells were harvested at the end of the 24 and 120 hour-treatment, and immediately frozen in liquid nitrogen for RNA extraction.

RNA isolation and Northern analysis: Polyadenylated RNA was isolated from all the treated and control cells described above using a Fastrack mRNA isolation kit obtained from Invitrogen Invitrogen, Inc. CA). All the steps were performed according to the instructions of the manufacturer.
Five to 10 μg of RNA were electrophoresed through 1.2% formaldehyde agarose gels and capillarily transferred to nylon membranes (Stratagene, La Jolla, CA), and fixed by U.V. cross linking. The TRPM2 cDNA plasmid was kindly provided by Dr. Martin Tenniswood, from the University of Ottawa (Ottawa, Canada). Human ICE cDNA was a gift from Dr. Yuan, from

Harvard Medical School (Boston, MA). Human bcl2 cDNA probe was obtained from the American Type Culture Collection (ATCC, Rockville, MD). The insert was further subcloned into p-bluescript vector in the orientation of T3 promoter and was used for preparing riboprobe. All other probes used in this study were purchased from the ATCC. The probes were labeled with (α -^{32}P) CTP by random primer labeling kit (Gibco, BRL, Gaithersburg MD). Riboprobes were prepared for the detection of bcl2 and TGF-β by *in vitro* transcribing the linearized template DNA with (α-^{32}P) UTP and then purified by the Biospin column (Biorad., Richmond, CA). The nylon membrane blot was prehybridized, hybridized in hybridization solution containing 50% formamide, 5xDenhardt's reagent, 2xSSC, 0.5%SDS and 100 μg of salmon sperm DNA at 42^{0}C overnight. Then membranes were washed twice in 2XSSC, 0.1%SDS at room temperature for 15 min, 1XSSC, 0.1% SDS at 55^{0}C, followed by 0.1XSSC, 0,1% SDS at 60^{0}C and exposed to a Kodak X-omat film with an intensifying screen at -70^{0}C. Relative mRNA contents of each experimental group were determined by scanning laser densitometry (Molecular Dynamics, Sunnyvale, CA). Northern blots were stripped and then reprobed sequentially with additional probes. The human β-actin probe was employed as a control for verifying load consistency and RNA integrity. Densitometric results were normalized by expressing the units obtained for a specific transcript relative to the units obtained for -actin transcript. The expression of -actin mRNA was not affected by any of the treatments applied to the cells in this study.

Differential Display

Differential display was performed essentially as described by Liang and Pardee (122). Total RNA was isolated from both treated and control cells and then treated with DNAse I using the Message Clean Kit (GenHunter, Nashville, TN) to remove genomic DNA. Three one-base anchored oligodeoxy thymidylic acid primers (5'-AAGCT$_{11}$A-3', 5'-AAGCT$_{11}$ C-3' and 5'-AAGCT$_{11}$G-3') were used to reverse transcribe total RNA into first-strand cDNA, which were amplified subsequently by PCR using arbitrary upstream primers H-AP8 (5'-AAGCTTTTACCGC-3'), H-AP9 (5'AAGCTTCATTCCG3'), HAP10 (5'AAGCTTCCACGTA3'), HAP11 (5'AAGCTTCGGGTAA-3'), H-AP12 (5'-AAGCTTGAGTGCT-3'), H-AP13 (5'-AAGCTTCGGCATA-3') and H-AP14 (5'-AAGCTTGGAGGCTT-3') (RNA Image Kit GenHunter, Nashville, TN). Polymerase chain reaction (PCR) was performed following the manufacturer's instructions. The resulting PCR products were fractionated on a 6% denaturing polyacrylamide DNA sequencing gel using [^{33}P] dATP (2000 Ci/mmole). The gels were then dried and exposed to film. The identity of differentially expressed bands was confirmed on northern blots using both total and polyadenylated RNA. The bands that were unique in expression were cut out from the gel, eluted and amplified by PCR using the same set of primers. Specific probes were generated by labeling reamplified or cloned cDNA fragments with [α-^{32}P] dCTP (random primer labeling kit, GIBCO, BRL).

Effect of Roscovitine and Resveratrol on the Proliferative Activity of Human Breast Epithelial Cells: MCF-7, MCF-10F, and MDA-MB-123, an estrogen receptor-negative human breast carcinoma cell line were routinely cultured in T75 flasks. Fully confluent cells were harvested, counted with hemocytometer and seeded into 96-well plates. After 24 hours in culture, the cells

were treated daily with 5, 10, 20, and 40 μg/ml roscovitine (Calbiochem, La Jolla, CA) and resveratrol per 5-40 μg/ml dissolved in DMSO and incubated for a length of time ranging from 24 to 144 hours. Control cells were incubated under identical conditions with the same volume of DMSO-containing medium.

RESULTS

Effect of hCG treatment on the proliferative activity of human breast epithelial cells: The quantification of the number of viable cells revealed that by 24 hr. of hormonal treatment there were no significant differences between control and hCG- treatedMCF-10F, BP1-E and T24 cells. MCF-7 cells, on the other hand, exhibited a significant inhibition (Fig. 1). By 120 hours in culture, control cells had shown a 20-60 fold increase in the number of viable cells, while hCG-treated MCF-10F, MCF-7, and BP1E-Tp cells did not differ in the number of viable cells from the values observed at 24 hr. T24 cells showed the same rate of cell growth in the control and in the hCG-treated cells (Fig.1).

Activation of programmed cell death genes by hCG treatment of MCF-10F cells: Northern blot analysis indicated that the 24 hr hCG treatment induced an elevation in the expression of TRPM2 by 2 fold, ICE by four fold, TGF- by 2.5 fold, c-myc by 2 fold, p53 by 3 fold, and $p21^{WAF1/CIP1}$ by 3 fold. The treatment did not modify their expression in comparison with its respective control cells(Fig. 2). By 120 hr of hormonal treatment the expression of all the apoptotic genes remained at the same levels observed at 24 hr. There was a slight increase in bcl2, and a decrease in the expression of c-myc, but the differences were practically negligible. Of interest was the observation that in the control cells an increment was observed with respect to the levels detected in the 24 hr control cells.

Activation of programmed cell death genes by hCG treatment of MCF-7 cells: The 24 hr hCG treatment of MCF-7 cells significantly induced an increase in the expression of TRPM2 (2.5 fold), TGF- (3 fold), c-myc (3.5 fold), p-53 (4 fold) and $p21^{WAF1/CIP1}$ (3 fold) transcripts as compared to the control cells (Fig. 3). The ICE transcript could not be detected in MCF-7 control cells, and hCG treatment did not modify its expression. The levels of bcl2 significantly declined after the hormonal treatment in comparison with control cells (Fig. 3). By 120 hr of hormonal treatment the levels of TRPM2, TGF-β, p53, and p-21 remained at the same levels observed after 24 hr treatment. The differences with their respective controls, however, had disappeared because of the elevation in the expression of these genes in control cells. The expression of bcl2 was more intense in the 120 hr than in the 24 hr control, and hCG treatment almost completely inhibited its expression.

Activation of programmed cell death genes by hCG treatment of chemically transformed human breast epithelial cells: The 24 hr hCG treatment of the transformed human breast epithelial cells BP1-E produced a significantly increase in the expression of ICE by greater than two fold, but increased only slightly the expression of TRPM2, TGF- β, p53, and $p21^{WAF1/CIP1}$ genes, whereas the expression of bcl2 and c-myc was down-regulated by the treatment (Fig. 4). Very little change in gene expression was observed in the 120 hr with respect to the 24 hr hCG-treated cells, but a notable increase in the expression of TRPM2, ICE, and $p2^{WAF1/CIP1}$ was

observed in control cells, in which the level of expression of these genes was similar to those of hCG-treated cells.

The hormonal treatment of BP1-E-Tp cells for 24 hr increased 2 fold the expression of TRPM2 (Fig. 5). This increase was less pronounced in BP1-E cells. The expression of bcl2 slightly decreased, and that of c-myc, TGF- β, and p-53 was not significantly modified in any of the cell lines in comparison to their respective controls. $P21^{WAF1/CIP1}$, on the other hand, was significantly increased (2.2 fold) in BP1E-Tp cells (Fig. 5). The 120 hr treatment induced changes similar to those observed in BP1E cells, namely increased expression of p53 and $p21^{WAF1/CIP1}$ in treated cells, and increased expression of TRPM2, and ICE in both control and treated cells.

Effect of hCG treatment on the expression of programmed cell death genes in T24 cells: The 24 hr treatment of T24 cells with hCG did not alter significantly the expression of TRPM2, bcl2, p53 and c-myc, but it induced a slight increase in the expression of TGF- and $p21^{WAF1/CIP1}$(Fig. 6). By 120 hr of treatment the expression of TRPM2 had increased in both control and hCG treated cells, and the same phenomenon was observed in the expression of bcl2 and TGF- β. The expression of c-myc, on the other hand, was lower in treated than in control cells, and that of p53 and $p21^{WAF1/CIP1}$ was almost indistinguishable. The postulated model of the effect of hCG on cell cycle arrest and apoptosis is shown in Figure 7.

Identification of new genes differentially expressed in breast carcinoma cells treated with human chorionic gonadotropin (hCG): We postulated that hCG exerts its protective effects on the mammary gland and on HBEC *in vitro* through activation or downregulation of specific genes. To prove this hypothesis we utilized a differential display (DD) technique for identifying and isolating candidate genes involved in this process. DD is a powerful technique for identifying genes differentially expressed in normal and cancer cells (122), tumor suppressor genes, such as integrin alpha-6, and genes potentially involved in chemoprevention (123).

Differential display was performed with RNA isolated from control cells and cells treated with hCG for 24 and 72 hours to identify transcriptionally regulated genes potentially involved in the inhibition of neoplastic cell growth induced by hCG. PCR amplifications were performed using 8 primer combinations. Three PCR products that were differentially expressed between control and hCG treated cells were identified; they were designated bands 19, 29, and 44 (Figure 8). Figures 8A and 8B indicate 2 representative PCR products that were reproducibly down regulated, and Fig. 8C shows one fragment that was upregulated by hCG treatment. The two PCR fragments, 19 and 29, were markedly reduced after a 24 hr hCG treatment in comparison with their respective controls (Figs. 8A and 8B), and this reduction persisted up to 72 hrs of treatment (results not shown). The third PCR fragment was upregulated by the 24 and the 72 hour-hCG treatment (Fig. 8C). The isolated differentially expressed bands were 265 to 350 bp in size.

Northern blot analysis using polyadenylated RNA confirmed the differential expression of the bands detected by PCR (Figs. 9A & B, 10A & B, and 11A & B). The three PCR bands were cloned and identified as clones 19, 29, and 44. Clone 19 showed two transcripts ranging from

1.2 to 1.8 Kb in size (Fig. 9A). This suggests that the two mRNAs were generated by alternate splicing of a common mRNA precursor. Both transcripts were significantly reduced at 24 hr. after hCG treatment. The 1.2 Kb transcript was more significantly reduced than the larger transcript (Fig. 9A). Similarly, clone 29 also showed 2 transcripts (Fig. 10A), which ranged from 0.6 to 1.2 Kb in size; both transcripts were significantly reduced by hCG treatment for 24 and for 72 hr. Clone 44 showed an increase in its expression of approximately 5 fold at 24 hr of hCG treatment, and it remained elevated up to 72 hr (Figs. 11A and 11B).

To date we have found no significant homology with any published genes for the sequences obtained from clones 19 and 29 cDNA fragments, suggesting that they represent previously unknown genes and therefore appeared to be novel. Clone 44, that was present in very small amount in control cells, was present in very high amount in cells treated with hCG for both 24 and 72 hrs (Figs. 11A and 11B). Sequence homology search of this clone showed 98% match with a novel gene expressed in apoptotic T-cell hybridoma cells treated with dexamethasone.

In the present study, partial nucleotide sequences of two genes (19 and 29 cDNA) showed no homology to published data bases, suggesting that they represent previously unknown genes and therefore appeared to be novel. Identification and characterization of these genes are important from a chemotherapeutic point of view. Studies from our laboratory have indicated the possibility that either the up- or down regulation of inhibins, or genes regulating apoptosis and cell cyclins might be involved in the differentiation of the lobular structures of the breast, which in turn, are important modulators of the susceptibility of this organ to undergo malignant transformation (124). Our *in vivo* studies have demonstrated that hCG inhibits tumor progression through the activation or downregulation of the same genes (101), thus indicating that hCG triggers similar responses in both normal and malignant breast epithelial cells from different species. Based upon our recent results we strongly believe that new genes are also involved in the regulation of these complex processes. Our observations indicate that hCG regulates gene expression in breast epithelial cells, thus providing a new tool for determining the precise role of specific genes through the isolation and analysis of their full length cDNAs. Furthermore, these results indicate that induction of apoptosis is an important parameter. Other genes that are affected by this hormonal treatment remain to be identified. However, the possibility exists that these unknown genes might be important in determining the chemopreventive effects of hCG.

Effect of Roscovitine on the Proliferative Activity of Human Breast Epithelial Cells: Exposure of MCF-7 cells to different concentrations of roscovitine ranging from 5 to 40 μg/ml reduced the growth rate of the cells in culture. It was evident from the growth curve in Figure 12 that cells exposed to 40 μg/ml roscovitine failed to proliferate in all time points examined. Compared to the controls, cells treated with 5 μg/ml grew exponentially, but at a reduced rate, while cells exposed to 10 and 20 μg/ml were unable to attain exponential growth. Data of roscovitine effect on the number of viable MCF-7 cells illustrated in Figure 13, demonstrates that the cdk inhibitor has a dose- and time-dependent effect on cell viability. There was no increase in cell numbers in the group of wells treated with 20 and 40 μg/ml roscovitine after 24 hours of exposure. Treatment of MCF-10F cells to 5, 10, 20 and 40 μg/ml roscovitine for 24, 48, 72, 96, and 120 hours showed that the proliferative activity of the cells was inhibited in a dose- and time-

dependent fashion. In comparison to the controls, exposure of the cells to 5 μg/ml resulted in a considerable decrease in the proliferative rate of MCF-10F cells after 48 hours of treatment (Fig. 14). Treatments with 10, 20, and 40 μg/ml resulted in abrogation of exponential cell growth. It is evident from Figure 15 that exposure to 5, 10, 20, 40 μg/ml for 24 hours had little or no effect on the number of viable MCF-10F cells. However, all concentrations of roscovitine markedly decreased the number of viable cells after a 72 hour treatment. These results suggest that the proliferative activity of this breast epithelial cell line was suppressed by roscovitine.

MDA-MB-231 control cells grew exponentially after a 48 hour incubation and attained confluence after 120 hours in culture. The growth kinetics of cells exposed to 5 μg/ml roscovitine was similar to that of control cells, but suppression of cell proliferation occurred after a 96-hour incubation (Fig. 16). Cells treated with 10 μg/ml attained exponential growth but with a reduced rate of proliferation, while 20 and 40 μg/ml treatments prevented the exponential growth of the cells. Moreover, reduction in number of viable cells was evident in the group of cells exposed to 40 μg/ml roscovitine after 24 hours, and this trend persisted throughout the entire duration of the study. It was also observed that the number of viable cells was severely decreased in the group that received 10, 20, and 40 μg/ml after 72 hours in culture (Fig. 17). Reduction in cell numbers in cells exposed to 5 μg/ml occurred only after 96 hours of roscovitine exposure.

Roscovitine Induces Cell Death and Morphological Changes Indicative of Apoptosis in MDA-MB-231 Breast Cancer Cells.

Although antiestrogens have proved beneficial for palliative care of breast cancer patients (125,126), it should be realized that only about 30% of breast cancer patients respond to endocrine therapy (127). Moreover, estrogen-responsive tumors initially respond to hormonal treatment but often progress to hormone-independent tumors and become resistant to endocrine therapy (128). Presently, therapeutic choices are limited for hormone-resistant and estrogen receptor-negative breast tumors which are often very aggressive (129). Thus, the treatment of highly invasive tumors which do not respond to endocrine therapy is a major challenge.

The lack of treatment choices for hormone-resistant tumors motivated our search for effective drugs that can be utilized for the clinical management of both hormone-responsive and non-responsive breast tumors. Very recently, *in vitro* screening for anticancer agents in our laboratory led to the discovery that roscovitine, a potent and selective inhibitor of cyclin-dependent kinases (cdks) (130,131), also inhibits the proliferative activity of human breast epithelial cells (132). Roscovitine is an olomoucine-related purine recently found to inhibit the kinase activity of cdc2/cyclin B, cdk2/cyclin A and cdk2/cyclin E complexes (133). Micromolar concentrations of roscovitine have been reported to prevent cell cycle progression of mammalian cells at the G1/S and G2/M checkpoints (134,135). Our experimental studies have demonstrated that roscovitine treatment prevented the exponential growth and decreased the number of viable cells in both estrogen receptor-positive and estrogen receptor-negative breast cancer cells (132). Since the antiproliferative effect of roscovitine is independent of the estrogen receptor status of the breast cancer cells, this potent cdk inhibitor is a potential pharmacological agent for the treatment of both hormone-responsive and non-responsive breast cancer cells. To facilitate the

translation of the antiproliferative efficacy of roscovitine to the clinical setting, a more detailed study to elucidate the mechanism(s) responsible for its growth inhibitory effect is warranted.
As illustrated by the growth curve in Figure 18, MDA-MB-231 control cells started to grow exponentially after 48 hours in culture and attained confluence after another 100 hours incubation. Compared with the controls, it is evident that 10 μg/ml roscovitine prevented the exponential growth of the cells. When the cells were exposed daily to roscovitine for 96 hours, washed and treated with the culture medium without roscovitine for 144 hours, the cells failed to grow despite the absence of the drug. These results demonstrate that the growth inhibitory effect of roscovitine on MDA-MB-231 breast cancer cells was irreversible.

As shown in Figure 19, MDA-MB-231 cells exposed to culture medium without roscovitine remained in the lag phase of growth for 48 hours, grew exponentially for about 100 hours and became fully confluent after 168 hours in culture. In contrast, daily treatment with 10 μg/ml roscovitine prevented the exponential growth of the cells. When non-dividing confluent cells were treated with roscovitine after 168 hours in culture, a time-dependent inhibition of cell growth was observed after 24 hours treatment. Compared with the controls, the number of viable cells measured by the WST-1 colorimetric assay was reduced by about 50% at the end of the experiment. Because the reduction of WST-1 Reagent to formazan product requires viable cells with functional mitochondrial dehydrogenases (136), these results suggests that roscovitine is cytotoxic to confluent or resting cells.

Measurement of BrdU incorporation during DNA synthesis by proliferating MDA-MB-231 breast cancer cells showed that roscovitine inhibited DNA synthesis in a dose-dependent manner. As illustrated in Figure 20, treatment of the cells with 1 μg/ml roscovitine had no measurable effect on BrdU incorporation compared with the controls but exposure of the cells to 5 μg/ml reduced BrdU incorporation by about 25%. Furthermore, exposure of the cells to 10 μg/ml inhibited DNA synthesis by 60% while the inhibition induced by treatment of the cells with 20 μg/ml roscovitine was about 90%. These results demonstrate that roscovitine acts as an anti-mitogenic agent in MDA-MB-231 breast cancer cells.

Differences in cell morphology were observed between roscovitine-treated and control MDA-MB-231 cells by light microscopy after staining with the Leukostat reagent. The most conspicuous changes observed in roscovitine-treated cells included cell shrinkage and extensive detachment of the cells from the cell culture substratum. These changes, which are characteristics of apoptotic cell death (137), became visible after 24 hours of roscovitine treatment, but were absent in control cells. The morphological changes became more remarkable with increased time of drug treatment. These observations suggested that cells treated with roscovitine detached from the substratum and died by apoptosis.

The occurrence of apoptosis was further verified by Hoechst staining which detects chromatin condensation, one of the hallmarks of apoptotic cell death (138). Some differences were observed in the nuclei of roscovitine-treated and untreated MDA-MB-231 breast cancer cells after staining with Hoechst 33342. The Hoechst 33342 dye stained morphologically normal nuclei dimly blue whereas roscovitine-treated cells demonstrated bright blue and smaller nuclei. These changes in nuclear morphology, which were initially observed after 24 hours of

roscovitine treatment, and increased thereafter, reflected chromatin condensation and nuclear shrinkage. These results demonstrate that roscovitine induces morphological changes characteristic of apoptotic cell death.

Because early breakdown of actin microfilaments has been reported to be a prerequisite for cell shape alterations and cell death in mammalian cells (139), we investigated the effect of roscovitine treatments on the organization of actin microfilaments in MDA-MB-231 breast cancer cells. Staining of the cells with TRITC-labeled phalloidin showed that DMSO- treated control cells exhibited a well-defined F-actin network that was mainly organized into stress fibers. Exposure of the cells to 10 μg/ml roscovitine resulted in disruption of the actin microfilament network. The time course of cytoskeletal modifications which was examined by fixing cells at various time intervals after roscovitine treatments revealed that distinct changes occurred in the architecture of actin microfilaments. The actin fibers of roscovitine-treated cells were disorganized, disassembled, or disrupted. These results clearly indicated that roscovitine induces the reorganization of actin microfilament architecture in MDA-MB-231 breast carcinoma cells.

Our data indicate that roscovitine induces an irreversible inhibitory effect on the proliferation of MDA-MB-231 human breast carcinoma cells. It is evident from the results presented herein that cells exposed to 10 μg/ml roscovitine lose their capability to proliferate after 96 hours treatment with the drug. This finding suggests that this selective inhibitor of cyclin-dependent kinases (cdks) has the potential to irreversibly suppress the growth of breast tumors. From a therapeutic point of view, the clinical relevance of this observation is that roscovitine does not need to be continuously present in the system in order to be effective in suppressing tumor growth. In addition to its irreversible effect on cell growth, a substantial reduction in the number of viable cells was observed in confluent nondividing cells treated with roscovitine, results that suggest that the antiproliferative effect of this compound may be partly due to its ability to induce cell death. Moreover, the measurement BrdU incorporation revealed that roscovitine inhibited DNA synthesis in MDA-MB-231 breast cancer cells. The inhibition of DNA synthesis by roscovitine is probably due to its direct effect on the DNA replication machinery, as suggested in a recent report that showed that DNA synthesis was suppressed by roscovitine in homogenized tissues of rat cerebral cortex (140).

The irreversible cytotoxic effect exerted on the cells by the drug, coupled with the finding that this purine analog induced cellular shrinkage and chromatin condensation, indicated that apoptosis was induced by roscovitine in MDA-MB-231 breast cancer cells. Our analyses of roscovitine-treated cells by both light and fluorescence microscopy are the first evidence demonstrating that morphological changes characteristic of apoptotic cell death was induced by this cdk inhibitor. Our current results suggest that inhibition of cdk2/cyclin E activity may trigger apoptosis in breast cancer cells. Growing evidence now indicate that the efficacy of many anticancer drugs is related to their ability to induce apoptosis (141), as such, induction of apoptosis by roscovitine may open new strategies for improving breast cancer therapy and prevention.

Analysis of actin cytoskeleton of MDA-MB-231 human breast cancer cells with TRITC-

conjugated phalloidin revealed that actin filaments of cells that were not treated with roscovitine were organized into a dense, dynamic meshwork of actin fibers, while the actin fibers of roscovitine-treated cells were either disorganized, disassembled, or disrupted. These observations suggest that roscovitine promotes the breakdown of actin microfilaments. To our knowledge, this is the first demonstration that roscovitine causes the reorganization of actin cytoskeleton. Although the mechanism of action of roscovitine on the cytoskeleton is not yet known, the dramatic effect of the drug on actin filaments indicates that this cdk inhibitor may be involved in cytoskeletal regulation, possibly by reducing the polymerization of actin microfilaments. Recent reports have shown that the reorganization of actin cytoskeleton induced by extracellular factors requires phosphatidylinositol 3-kinase (PI3-kinase) activation and subsequent rise in the concentration of intracellular free calcium (142,143). Thus, it is very likely that the reorganization of actin microfilament induced by roscovitine in our model system may involve the activation of the PI3-kinase signaling pathway and induction of calcium influx. In addition, roscovitine may prevent the assembly of actin fibers by modulating the expression and/or activity of Rho GTPases, which have been reported to be involved in the regulation of actin microfilament organization and other associated activities (144-146). Disruption of actin microfilament architecture by roscovitine has some biological implications. Considering the role played by actin microfilaments in various aspects of cellular physiology such as cell-cell interactions, cell migration, proliferation and secretion (146), it can be argued that all these cellular activities could be affected in breast tumors following roscovitine treatment.

Another notable observation from our morphological analysis was the extensive detachment of cells from the cell culture substratum after exposure to roscovitine. Twenty fours after treatment, a progressive loss of cell attachment was observed in roscovitine-treated cells, which was not observed in the untreated set of cells. In adherent cells, detachment from the substratum is associated with morphological changes characteristic of apoptosis such as cellular shrinkage and chromatin condensation (147). Recent evidence suggests that cellular attachment to the substratum is mediated by the interactions of integrins with extracellular matrix (ECM) components such as fibronectin, collagen and vitronectin (137). Binding of integrins to these adhesion molecules results in activation of focal adhesion kinase (FAK)(148,149) accompanied by phosphorylation and recruitment of a number of related cytoskeletal and signaling molecules, thereby transducing anchorage and survival messages to the nucleus (150-152). Conversely, uncoupling of integrins from ECM proteins leads to disruption of integrin-mediated signal transduction, inactivation of FAK, detachment of cells from the extracellular matrix, and apoptotic cell death (153-155). Our data suggest that following roscovitine treatments, MDA-MB-231 cells detach from cell culture substratum and die via apoptosis. This notion is consistent with previous reports that demonstrated that cells deprived of matrix attachment underwent apoptosis (156,157). Thus, the extensive detachment of cells from the cell culture substratum and apoptotic cell death observed in our experimental system might be due to uncoupling of integrin-mediated signaling and/or disruption of cell-matrix interactions induced by roscovitine. In addition to facilitating apoptosis, the loss of adhesion induced by this cdk inhibitor may deny the cells anchorage and traction necessary for growth and migration; and thus prevent breast cancer invasion and metastasis, the major cause of death in breast cancer patients. Since adhesion and invasion are crucial to the initiation of metastatic growth (158), further studies on the effect of roscovitine on cell adhesion to extracellular matrix components as well as

the anti-invasive potential of the drug could be extremely rewarding. Such studies are now going on in our laboratory.

Antiproliferative Effect of Synthetic Resveratrol on Human Breast Epithelial Cells.

It has been suggested that clinical chemoprevention, a term that refers to the use of drugs, hormones or dietary compounds to block or reverse the initiation, promotion, and progression of cancer development, may be a plausible strategy for breast cancer control (159-161). Evidence from epidemiological and experimental studies indicates that natural constituents present in the diet can act as chemopreventive agents to inhibit mammary carcinogenesis (162-165). One of such compounds is resveratrol, a trihydroxystilbene unique to red wines and also present in grapes, berries, and peanuts (166,167). The cancer preventing activity of resveratrol was demonstrated in a recent study which showed that this natural constituent of the human diet inhibits cellular events associated with tumor development (167). Although this phytoalexin is believed to be anticarcinogenic, the chemopreventive potency and proliferative effect of both natural and synthetic resveratrol on breast cancer is not known. Thus, the present study was designed to study the effect of synthetic resveratrol on the proliferative capacity of malignant and non-malignant human breast epithelial cells in culture.

Effect of resveratrol on the in vitro growth of MCF-7 cells. Exposure of MCF-7 breast carcinoma cells to concentrations of resveratrol ranging from 5 to 40 μg/ml led to a decrease in the rate of exponential growth of the cells in culture. As revealed by the growth curves in Figure 21, both treated and untreated cells remained in the lag phase of growth for about 48 hours post-plating before exponential growth occurred. The control cells grew logarithmically for another 72 hours before reaching confluence. Although cells exposed to 5 μg/ml resveratrol attained exponential growth, their rate of growth was moderately reduced by the treatment. Cells exposed to the 10, 20, and 40 μg/ml doses failed to attain exponential growth. The effect of resveratrol on the viability of MCF-7 cells is illustrated in Figure 22. The data clearly demonstrate that this phytoalexin has an inhibitory effect on the viability of MCF-7 breast carcinoma cells which was more remarkable when 10, 20, and 40 μg/ml doses were used. The inhibition observed after 24 hours of treatment was maintained up to 144 hours.

Effect of resveratrol on MCF-10F cell growth. Treatment of MCF-10F cells with 5, 10, 20, or 40 μg/ml resveratrol for 24, 48, 72, 96, and 120 hours showed that their proliferative activity was inhibited in a dose- and time-dependent manner. Like the controls, cells exposed to 5 μg/ml grew exponentially but at a reduced rate, treatment with 10μg/ml produced a more intense inhibition while treatment with 10, 20, and 40 μg/ml completely inhibited the exponential growth of the cells (Figure 23). As shown in Figure 24, resveratrol did not affect the viability of MCF-10F cells for the first 24 hours of treatment at any of the doses tested. A dose- and time-dependent effect became clearly evident after 48 hours of treatment and was maintained until 120 hours.

Effect of resveratrol on the growth of MDA-MB-231 cells. Exposure of MDA-MB-231 breast carcinoma cells to concentrations of resveratrol ranging from 5 to 40 μg/ml reduced the growth rate of the cells in culture. As shown by the growth curve in Figure 25, the growth kinetics of

cells exposed to 5 μg/ml was similar to that of the control cells for the first 60 hours of treatment, the suppression of cell proliferation becoming evident thereafter. Treatment with 10 and 20 μg/ml doses substantially reduced the exponential growth of the MDA-MB-231 cells, while cells treated with 40 μg/ml resveratrol failed to proliferate at all time points examined. Figure 26 demonstrates that 24 hours exposure of MDA-MB-231 breast carcinoma cells to 5, 10, 20, and 40 μg/ml resveratrol had no effect on the viability of the cells. However, treatment with 10, 20, and 40 μg/ml markedly decreased the number of viable cells after 72 hours in culture. Compared with the controls, 5 μg/ml resveratrol substantially reduced the number of viable cells only after 120 hours of treatment.

Presently the mechanisms responsible for the antiproliferative effect of resveratrol remain largely obscure, however, the substantial inhibition of cell viability shown in our experiments suggests that the inhibitory effect of this common constituent of the human diet may be related to its ability to induce cell death by apoptosis, a phenomenon associated with the effects of several anti-cancer drugs (168-171). The antiproliferative effect of resveratrol observed in this study is consistent with the activity of other anti-cancer drugs, such as bleomycin, doxorubicin, cisplatin, and methotrexate, which have been recently reported to induce apoptosis in human hepatoma cells (172). Furthermore, since apoptosis provides an efficient mechanism for eliminating excessive or unwanted cells during carcinogenesis (172,173), the decreased number of viable human breast epithelial cells seen after resveratrol treatment might be due to its ability to eliminate cells that are in the process of neoplastic transformation by inducing apoptotic cell death.

Recent evidence suggests that resveratrol inhibits cellular events involved in the initiation, promotion, and progression of tumors (167). Resveratrol is believed to exert its influence on the carcinogenic process through inhibition of free-radical formation and cyclooxygenase (COX-1) activity, as well as induction of terminal differentiation which correlates with anti-tumor initiation, promotion, and progression respectively (167). It is very likely that the induction of growth arrest by resveratrol in our experimental system is mediated by these anti-tumor cellular processes. Induction of cellular differentiation by anti-tumor and chemopreventive compounds extracted from plants has also been found to suppress uncontrolled proliferation in HL-60 leukemia cells (174). These reports, combined with our current data, suggest that besides the induction of cell death, resveratrol could also induce differentiation of human breast cells. The remarkable antiproliferative effect of resveratrol demonstrated in this study suggests that this dietary compound could be a useful anti-tumor agent for breast cancer chemoprevention. However, *in vivo* studies are warranted to further establish the usefulness of resveratrol as a cancer chemopreventive agent.

DISCUSSION

The results of the present investigation demonstrate that treatment of human breast epithelial cells with the human placental hormone chorionic gonadotropin obtained from the urine of pregnant women, which is clinically used for induction of ovulation, among other applications (175), inhibits the proliferative activity of the cells and induces activation of genes controlling programmed cell death. This effect was observed only in HBEC, either the normal immortalized

MCF-10F, or the chemically transformed pre- and tumorigenic cells BP1E and BP1E-Tp, respectively, and the malignant metastatic MCF-7 cells, the urothelial cells T24 were not affected by this treatment. The difference in responsiveness observed between HBEC and urothelial cells at the level of activation of the genes that control programmed cell death coincides with the selective inhibitory effect of hCG on *in vitro* HBEC proliferation, which is not observed in T24 cells (29). This specificity of action might be attributed to the presence of a receptor to hCG that we have detected in HBEC (unpublished observations), and that has been recently reported in rat mammary epithelial cells (176). Although all HBEC are growth inhibited and express activation of programmed cell death under hCG treatment, the response of the cells to the hormonal treatment varies depending upon the basic biological conditions of the cells. For example, most of the genes controlling programmed cell death were activated in the immortalized, but otherwise normal HBEC MCF10-F cells, whereas the chemically transformed cells BP1E and BP1E-Tp and the metastatic human breast carcinoma cell line MCF-7 exhibited differences which might be related to variations in the pathway of activation of programmed cell death (Fig. 7).

In our experimental model, activation of TRPM2 and TGF- g enes by hCG treatment occurred in MCF-10F and MCF-7 cells, but not in the chemically transformed HBEC and the T24 cell lines, an observation that supports the concept that activation of these two genes might be dependent on specific cell characteristics. Induction of TRPM2 transcript has been shown during chemotherapeutic regression of a mouse bladder tumor (177). Although enhanced expression of TRPM2 and TGF- genes has been reported in regressing human breast cancer cells following estrogen ablation and in prostatic tumors after hormone withdrawal (178,179), we have observed that in the rat mammary carcinoma model, activation of TRPM2 occurs under maximal hormonal stimulation (180). TRPM2 has been reported to be stimulated in MCF-7 cells by 1,25-dihydroxyvitamin D3, which also inhibits cell proliferation (181).

Another gene that has been shown to be relevant in the induction of apoptosis is the ICE gene. ICE belongs to a protease family, which includes ICE and CPP32/Yama, or apopain (42-46). The role of ICE in driving the cells to apoptosis has been confirmed by the observations that its inhibition and/or mutation inhibits apoptosis induced by various stimuli (44,51,182). In our experimental system, the expression and behavior of the ICE gene under hCG treatment differs among the different cell lines tested. It is significantly increased in MCF-10F and in the transformed BP1-E and BP1E-Tp cells, but it is barely expressed and does not respond to the hormonal treatment in MCF-7 cells. In T24 cells, on the other hand, the gene is present, but its expression is not modified by hCG treatment.

Several lines of evidence indicate that induction of apoptosis can be mediated by both p53 and c-myc (63), which are the major players in the context of growth arrest and apoptosis. It has also been demonstrated that c-myc mediated apoptosis requires functional p53 (183,184). Furthermore, recent evidence indicates that induction of apoptosis can be mediated by the tumor suppressor p53 through its downstream target gene p21 $^{WAF1/CIP1}$ (63-65). We have found that under hCG treatment the expression of p53and c-myc is significantly increased in both MCF-10F and MCF-7 cells, but it is not modified in either BPlE, BPlE-Tp or T24 cells. These observations are relevant to the light that deregulated expression of c-myc is often associated with an increased incidence of cell death, usually by apoptosis. It has been suggested that cell

proliferation and cell death are tightly coupled or overlapping processes that are both driven by c-myc, although once they are established, cell growth and cell death are independently modulated by other genes and other external factors (34,35,64,185). C-myc mediated apoptosis requires functional p53 (183,184,186), as demonstrated by the induction of apoptosis by activation of c-myc in p53 +/+ fibroblasts, which is preceded by stabilization of p53, whereas in quiescent p53-null fibroblasts c-myc activation did not result in apoptosis (183). Whereas in mammary and other epithelial cells in culture, both p53 dependent and p53 independent apoptosis pathways have been identified (52,180,184, 186-188), our observations that p53 was significantly activated by hCG indicated that this gene is involved in the process of programmed cell death, in association with the inhibition of in vitro cell proliferation. Using Northern blot analysis, we have further shown profound induction of $p21^{WAF1CIP1}$ mRNA in all the cell lines treated with hCG. Based upon these observations it can be concluded that hCG first arrests the progression of cell cycle by inducing p53 and its target gene $p21^{WAF1CIP1}$ and then proceeds towards apoptosis (Fig.7). In the case of chemically transformed cell lines, we did not find any change in the levels of p53, but there was a profound induction of WAFl/ClPl mRNA. The possibility exists that WAF1 was induced by hCG independently of p53, as it has been shown in other systems (189).

The molecular details of p53-induced cell death are not fully understood, but conserved features include activation of proteases of ICE class (190). This mechanism is supported by our findings that hCG treatment induces an increase in the expression of both p53 and ICE in MCF-10F cells. This postulate, however, is not supported in MCF-7 cells, in which ICE is barely expressed, and it is not modified by the hCG treatment, supporting the concept that each cell type may respond differently to hormonal stimuli in the activation of these genes. Another possible involvement of p53 in apoptosis is the regulation by members of the bcl2 multiprotein family (191-194). Some of the members of the bcl2 family which are overproduced in a variety of human cancers (i.e., bcl2, bcl-XL) are blockers of cell death, while others, such as Bax and bcl-XS, are promoters of apoptosis, and their levels might be reduced in some types of cancers (191-194). In the present study we found that hCG treatment did not modify the expression of bcl2 in MCF-10F cells, but it induces its down-regulation in MCF-7 cells. The fact that p53 and bcl2 expression is differently modulated by the hormonal treatment is a strong indication that alternative pathways might be operational in the activation of programmed cell death genes by hCG. In the performance of these studies we observed that control cells, after 120 hours in culture exhibited an elevation in the level of expression of apoptotic genes which was similar to the levels observed in the 24 hr hCG-treated cells, indicating that hCG accelerates the process of gene activation that has been reported to be associated with confluence (195). Our observations led us to conclude that a 24 hour treatment of immortalized, chemically transformed and malignant human breast epithelial cells with hCG activates programmed cell death genes even before an arrest of cell growth has become evident. Of relevance is the fact that hCG that in vivo acts as a preventive and tumoristatic agent (19,86,87), and an inhibitor of in vitro cell proliferation (29,81,82,87), may utilize different pathways for activating programmed cell death genes, depending upon the degree of expression of neoplastic phenotypes.

Our study of the proliferative responses of different human breast epithelial cell lines to rescovitine, a potent chemical inhibitor of cdc2 and cdk2 utilizing MCF-7, an estrogen receptor-positive human breast carcinoma cell line (80,81), MCF-10F, an estrogen receptor-negative

immortal human breast epithelial cell line (82) and MDA-MB-231, a highly malignant estrogen receptor-negative human breast epithelial cell line demonstrated that roscovitine suppressed the proliferation of the three breast epithelial cell lines in a dose- and time-dependent fashion. Cell proliferation measurements with the WST-1 colorimetric assay revealed that roscovitine treatment reduced the number of viable cells and prevented the exponential growth of all the cell lines examined.

The cellular and molecular mechanisms responsible for the antiproliferative effect of roscovitine on human breast epithelial cells is not yet defined, however, its ability to inhibit cdk kinase activity by competition for ATP (77) may prevent the phosphorylation of pRb and thus inhibit cell growth. The possibility that roscovitine effects is mediated by estrogen receptors is unlikely because data from the present study indicate that similar growth responses were elicited from both estrogen receptor-positive and estrogen receptor-negative cell lines by roscovitine. Evidence from classical enzymological studies have elegantly demonstrated that roscovitine selectively inhibits the kinase activity of cdc2/cyclin B, cdk2/cyclinE and cdk2/cyclin A complexes (76,78). Since these activated cdk complexes are required for progression of cells through different phases of the cell cycle, it is conceivable that the antiproliferative of roscovitine effect may be due to its ability to block the cell cycle-propelling activity of the activated kinases and induce growth arrest.

Suppression of cdk2 kinase activity is one of he mechanisms utilized by proliferative signals to regulate cell cycle progression during G1. Previous studies had implicated $p21^{Cip1}$ and $p27^{Kip1}$ in mediating the effects of negative proliferative signals such as contact inhibition, TGF-, and DNA damage (196,197). Moreover, overexpression of $p21^{Cip1}$, a universal inhibitor of cyclin-dependent kinases has been shown to inhibit the proliferation of several mammalian cells (78). Our results indicate that roscovitine mimics the action of natural cdk inhibitors by inducing cell growth arrest in our experimental model system. The substantial reduction in the number of viable human breast epithelial cells observed in our experiments might also be due to the ability of this potent cdk inhibitor to induce physiological cell death. Evidence from recent studies have shown that olomoucine, and butyrolactone-1 also known to inhibit cdk activity, enhanced drug-induced apoptosis in human leukemia cells and murine mammary cell lines (198,199). Like roscovitine, olomoucine and butyrolactone-1 are thought to inhibit cdk1 and cdk2 protein kinase activity by competition with ATP (76,77). Thus, it is very likely that roscovitine treatment triggers apoptotic cell death in human breast epithelial cells. Data obtained from this study suggest that roscovitine is a potential antineoplastic agent. To facilitate the translation of roscovitine's efficacy to the clinical setting, a more detailed study to elucidate the mechanisms responsible for the antiproliferative effect of this potent cdk inhibitor would be necessary. Such experiments are now underway in our laboratory. The relevance of our findings lies in the potential use of hCG as a chemopreventive and chemotherapeutic agent in breast cancer, utilizing the detection of activation of programmed cell death genes as an early end point in the action of this hormone on the target tissues.

CONCLUSIONS

Our observations led us to conclude that treatment of immortalized, chemically transformed and

malignant human breast epithelial cells with hCG activates programmed cell death genes even before an arrest of cell growth has become evident. Of relevance is the fact that hCG in vivo acts as a preventive and tumoristatic agent. *In vitro* it acts as an inhibitor of cell proliferation, an indication that this hormone might utilize different pathways for either activating programmed cell death genes or inhibiting specific cell cycle dependent kinases, depending upon the degree of expression of neoplastic phenotypes. The relevance of these findings lies in the potential use of hCG as a chemopreventive and chemotherapeutic agent in breast cancer, utilizing the detection of activation of programmed cell death genes as an early end point in the action of this hormone on the target tissues.

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APPENDIX

Legend of Figures

Figures 1-26

List of Publications and Meeting Abstracts

Legends of Figures

Figure 1. Effect of hCG treatment on cell growth. MCF-10F, MCF-7, BP1-E-Tp, and T24 cells were treated daily with 100 IU/ml hCG and harvested at 24 and 120 hours cell growth determination by WST-colorimetric assay. Control cells were treated with vehicle only. Values represent the mean number of viable cells (x 1000) ±SD of three wells from two experiments.

Figure 2. Histogram showing the expression of TRPM2, ICE, bcl-2, TGF-β, c-myc, p53 and p21 mRNA relative to their respective controls in MCF-10F cells treated with hCG for 24 hours. Relative mRNA content was determined by scanning laser densitometry of autoradiographs, and equalized by detection of β-actin.

Figure 3. Histogram showing the expression of TRPM2, bcl-2, TGF- β, c-myc, p53 and p21 mRNA relative to their respective controls in MCF-7 cells treated with hCG for 24 hours. Relative mRNA content was determined by scanning laser densitometry of autoradiographs, and equalized by detection of β-actin.

Figure 4. Histogram showing the expression of TRPM2, ICE, bcl-2, TGF- β, c-myc, p53 and p21 mRNA relative to their respective controls in BP1-E cells treated with hCG for 24 hours. Relative mRNA content was determined by scanning laser densitometry of autoradiographs, and equalized by detection of β-actin.

Figure 5. Histogram showing the expression of TRPM2, ICE, bcl-2, TGF- β, c-myc, p53 and p21 mRNA relative to their respective controls in BP1-E-Tp cells treated with hCG for 24 hours. Relative mRNA content was determined by scanning laser densitometry of autoradiographs, and equalized by detection of β -actin.

Figure 6. Histogram showing the expression of TRPM2, bcl-2, TGF- β, c-myc, p53 and p21 mRNA relative to their respective controls in T24 cells treated with hCG for 24 hours. Relative mRNA content was determined by scanning laser densitometry of autoradiographs, and equalized by detection of β-actin.

Figure 7. Postulated model of hCG-induced cell cycle arrest and apoptosis in human breast epithelial cells. In the presence of hCG for 24 hours breast epithelial cells the binding of the hormone to a newly discovered, putative membrane receptor, that triggers a cascade of programmed cell death gene activation through the cAMP-PKA pathway, as well as through activation of TGF- β. In the chemically transformed BP1-E and BP1-E-Tp cells in which TGF- β, c-myc and p53 are not activated, it is postulated that p21 activation might proceed through an alternative pathway, i.e., TF/DF (wavy arrow).

Figure 8a-c:Differential Display comparing RNAs from control and hCG treated MCF-7 cells. A and B: Autoradiogram of amplified [α-^{33}P] dATP-labeled PCR products are shown for a primer combination that identified two distinct fragments (arrows) which were downregulated by hCG treatment. The primer combination included H11A, G or C and HAP-9. Lanes 1 and 2: MCF-7 control cells, lanes 3 and 4, cells treated with hCG for 24 hr. C: Autoradiogram of amplified [α-

^{33}P] dATP-labeled PCR products for the primer combination H11A, G or C and HAP-11 that identified one fragment (arrow) upregulated by hCG treatment. Lane 1, control and lane 2, cells treated with hCG for 24 hr, lane 3, control, lane 4, cells treated with hCG for 72 hr.

Figure 9. A: Northern blot analysis of MCF-7 cells treated with hCG confirming gene expression for differentially displayed bands (clone 19). Probes were generated from the cloned fragments. β-actin m-RNA was used as a control for verifying the loading of RNA samples. Lane 1, Control cells (cont.); lane 2, MCF-7 cells treated with hCG for 24 hr.; lane 3, 72 hr control cells, and lane 4, MCF-7 cells treated with hCG for 72 hr. B: Histogram showing down-regulation of mRNA expression of clone 19 by treatment of MCF-7 cells with hCG. Abbreviations as per Figure 8A.

Figure 10. A: Northern blot analysis of MCF-7 cells treated with hCG confirming gene expression for differentially displayed bands (clone 29). Probes were generated from the cloned fragments. β-actin m-RNA was used as a control for verifying the loading of RNA samples. B: Histogram showing down -regulation of mRNA expression of clone 29 by treatment of MCF-7 cells with hCG. Abbreviations as per Figure 8A.

Figure 11. A: Northern blot analysis of MCF-7 cells treated with hCG confirming gene expression for differentially displayed bands in treated cells (clone 44). Probes were generated from the cloned fragments. β-actin m-RNA was used as a control for verifying the loading of RNA samples. B: Histogram showing increased mRNA expression of clone 44 by treatment of MCF-7 cells with hCG. Abbreviations as per Figure 8A.

Figure 12. Effect of roscovitine on growth kinetics of MCF-7 cells. Cells were grown in medium supplemented with 5% fetal bovine serum, exposed to different concentrations of roscovitine dissolved in DMSO and harvested at 24, 48, 72, 96, 120, and 144 hours. Cell proliferation was determined by WST-1 microplate assay. Values represent the mean ± SD of six wells from two experiments.

Figure 13. Effect of roscovitine on number of viable MCF-7 cells. Cells grown in medium supplemented with 5% fetal bovine serum were treated with different concentrations of roscovitine and harvested at 24, 48, 72, 96, 120, and 144 hours for WST-1 colorimetric assay. Values represent the mean ± SD of six wells from two experiments.

Figure 14. Effect of roscovitine on growth kinetics of MCF-10F cells. Cells were grown in medium supplemented with 5% horse serum and exposed to 5, 10, 20, and 40 μg/ml roscovitine and harvested at 24, 48, 72, 96, and 120 hours for WST-1 cell proliferation assay. Values represent the mean ± SD of six wells from two experiments.

Figure 15. Effect of roscovitine on number of viable MCF-10F cells. Cells grown in medium supplemented with 5% horse serum were treated with different concentrations of roscovitine and harvested at 24, 48, 72, 96, and 120 hours for WST-1 colorimetric assay. Values represent the mean ± SD of six wells from two experiments.

Figure 16. Effect of roscovitine on growth kinetics of MDA-MB-231 cells. Cells were grown in medium supplemented with 10% fetal calf serum, treated with different concentrations of roscovitine and harvested at 24, 48, 72, 96, and 120 hours for WST-1 cell proliferation assay. Values represent the mean ± SD of six wells from two experiments.

Figure 17. Effect of roscovitine on number of viable MDA-MB-231 breast carcinoma cells. The cells were cultured in medium supplemented with 10% fetal calf serum; exposed to different concentrations of roscovitine and harvested at 24, 48, 72, 96, and 120 hours for WST-1 colorimetric assay. Values represent the mean ± SD of six wells from two experiments.

Figure 18. Irreversibility of roscovitine effect. Three sets of MDA-MB-231 breast cancer cells were seeded into triplicate wells in 96-well plates. After 24 hours in culture, the controls were exposed daily to vehicle-containing medium while the second set was exposed daily to 10μg/ml roscovitine for 120 hours. The third set of cells were treated daily with 10μg/ml roscovitine for 96 hours, washed with serum-fee medium as indicated by the arrow, and then exposed to vehicle-containing medium for 144 hours. Cells were harvested daily for WST-1 assay. Values represent the mean ± SD of thee wells from two experiments.

Figure 19. Cytotoxic effect of roscovitine. Three sets of cells were seeded into triplicate wells in 96-well plates. After 24 hours in culture, the control cells were exposed daily to vehicle-containing medium while the second set was exposed daily to 10μg/ml roscovitine for 240 hours. The third set of cells was daily treated with vehicle-containing medium for 120 hours and then exposed to 10μg/ml roscovitine for another120 hours when the cells have ceased growing. Cells were harvested daily for WST-1 assay. Values represent the mean ± SD of three wells from two experiments.

Figure 20. Effect of roscovitine on DNA synthesis. MDA-MD-231 cells were seeded into triplicate wells in 96-well plates. After attachment, the cells were exposed to different concentrations of roscovitine and harvested at 48, 72, and 96 hours for BrdU incorporation assay. Values represent the mean ± SD of three wells from two experiments.

Figure 21. Effect of resveratrol on the growth kinetics of MCF-7 cells. Cells were grown in medium supplemented with 5% fetal bovine serum, and exposed to 5, 10, 20, or 40 μg/ml resveratrol dissolved in DMSO. Treated and control cells were harvested at 24, 48, 72, 96, 120, and 144 hours. Cell proliferation was determined by WST-1 microplate assay. Values represent the mean ± SD of six wells per dose per time point from two experiments.

Figure 22. Effect of resveratrol on number of viable MCF-7 cells. Cell viability was determined in cells grown and treated with resveratrol as described for Figure 17. Treated and control cells were harvested at 24, 48, 72, 96, 120, and 144 hours for WST-1 colorimetric assay. Values represent the mean ± SD of six wells from two experiments.

Figure 23. Effect of resveratrol on growth kinetics of MCF-10F cells. Cells were grown in medium supplemented with 5% horse serum and exposed to 5, 10, 20, or 40 μg/ml resveratrol

and harvested at 24, 48, 72, 96, and 120 hours for WST-1 cell proliferation assay. Values represent the mean ± SD of six wells from two experiments.

Figure 24. Effect of resveratrol on number of viable MCF-10F cells. Cells grown and treated with resveratrol as described for Figure 19 were harvested at 24, 48, 72, 96, and 120 hours for WST-1 colorimetric assay. Values represent the mean ± SD of six wells from two experiments.

Figure 25. Effect of resveratrol on the growth kinetics of MDA-MB-231 cells. Cells were grown in medium supplemented with 10% fetal calf serum, treated with 5, 10, 20, and 40 μg/ml resveratrol and harvested at 24, 48, 72, 96, 120 hours for WST-1 cell proliferation assay. Values represent the mean ± SD of six wells from two experiments.

Figure 26. Effect of resveratrol on number of viable MDA-MB-231 cells. The cells were cultured and treated with resveratrol as described in Figure 5. Treated and control cells were harvested at 24, 48, 72, 96, and 120 hours for WST-1 colorimetric assay. Values represent the mean ± SD of six wells from two experiments.

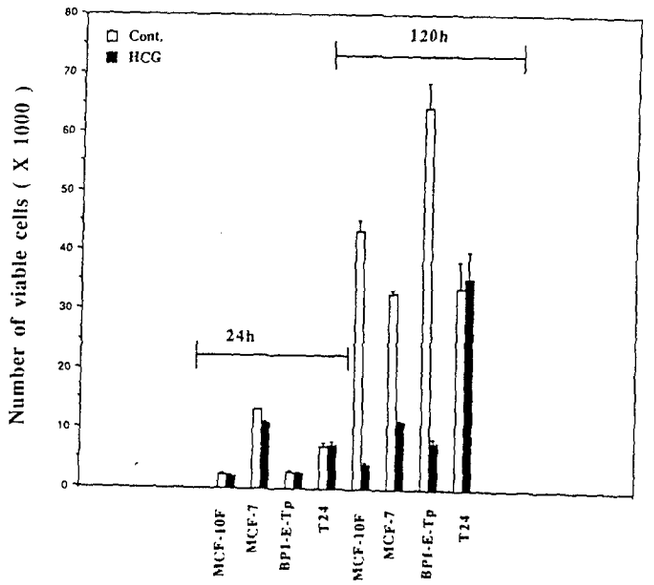
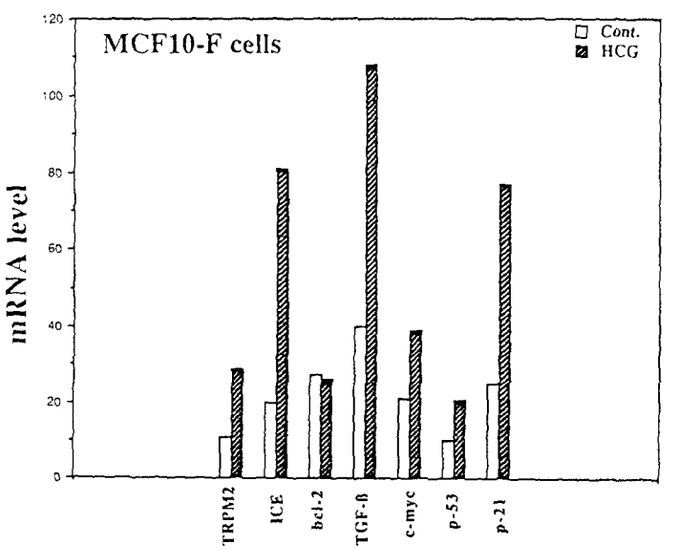


Figure 1



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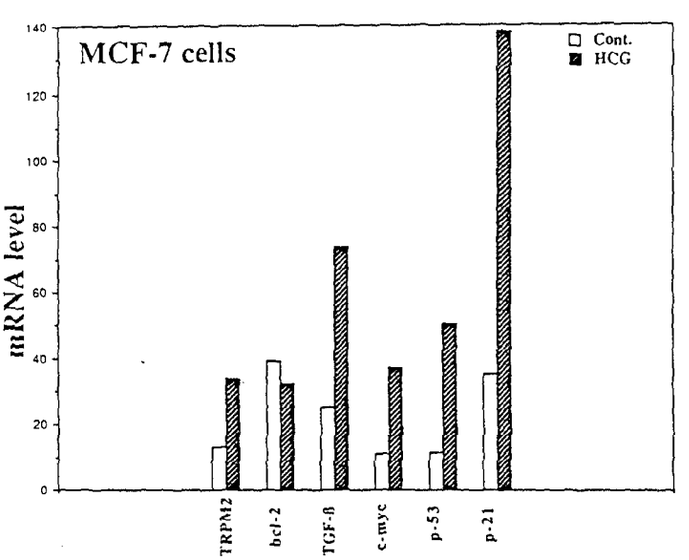

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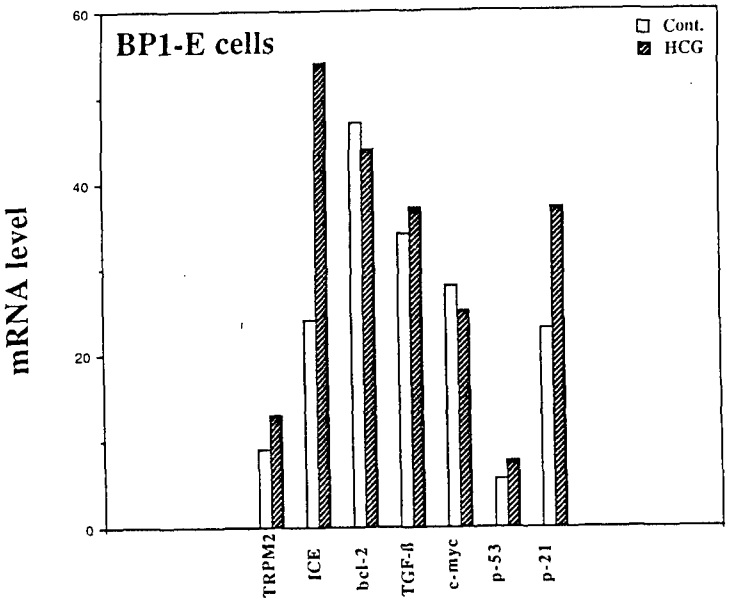

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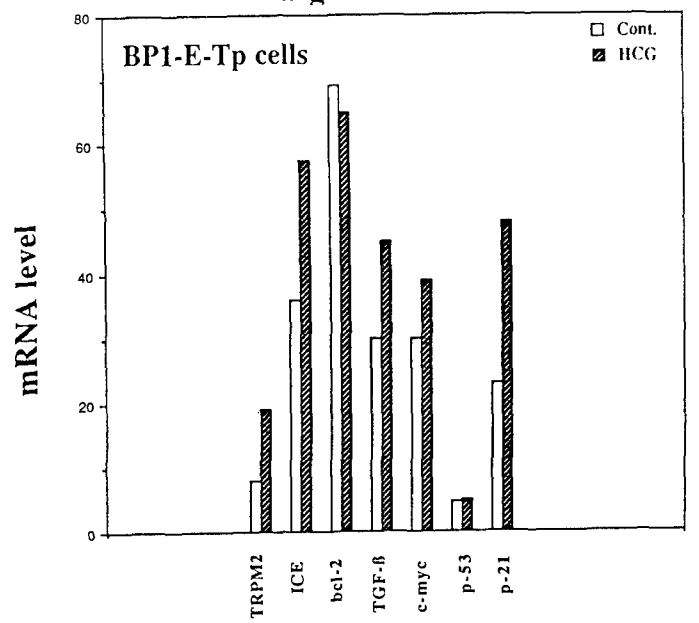

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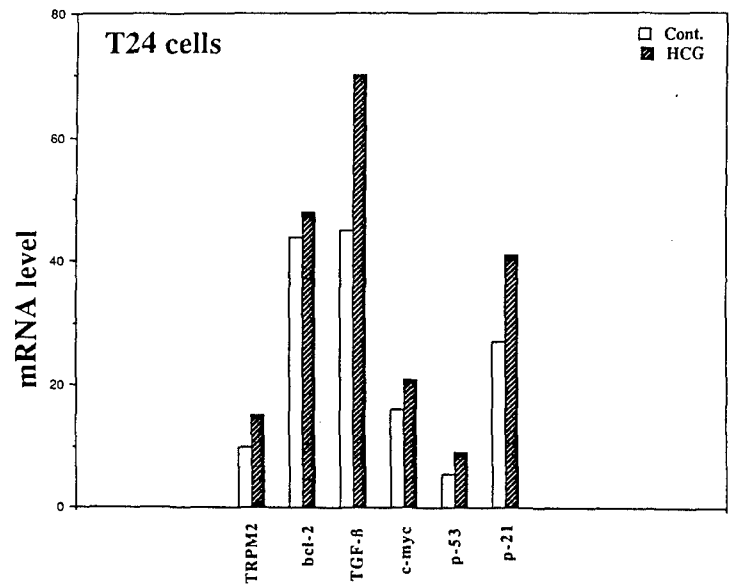

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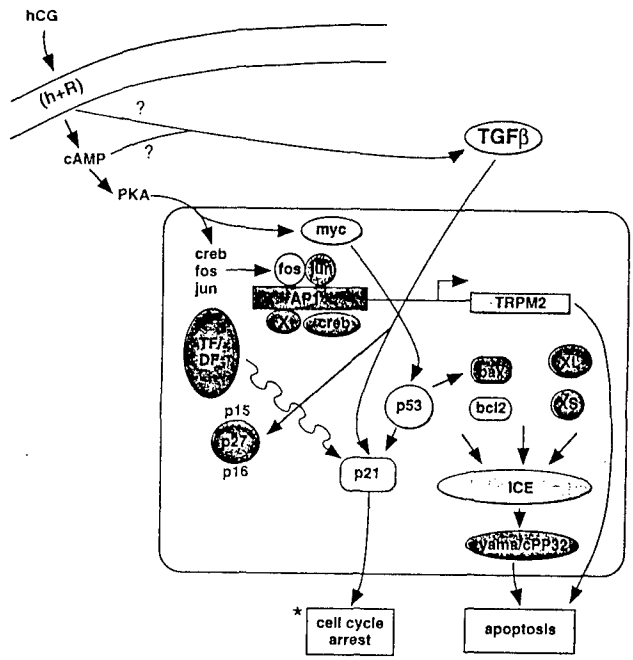

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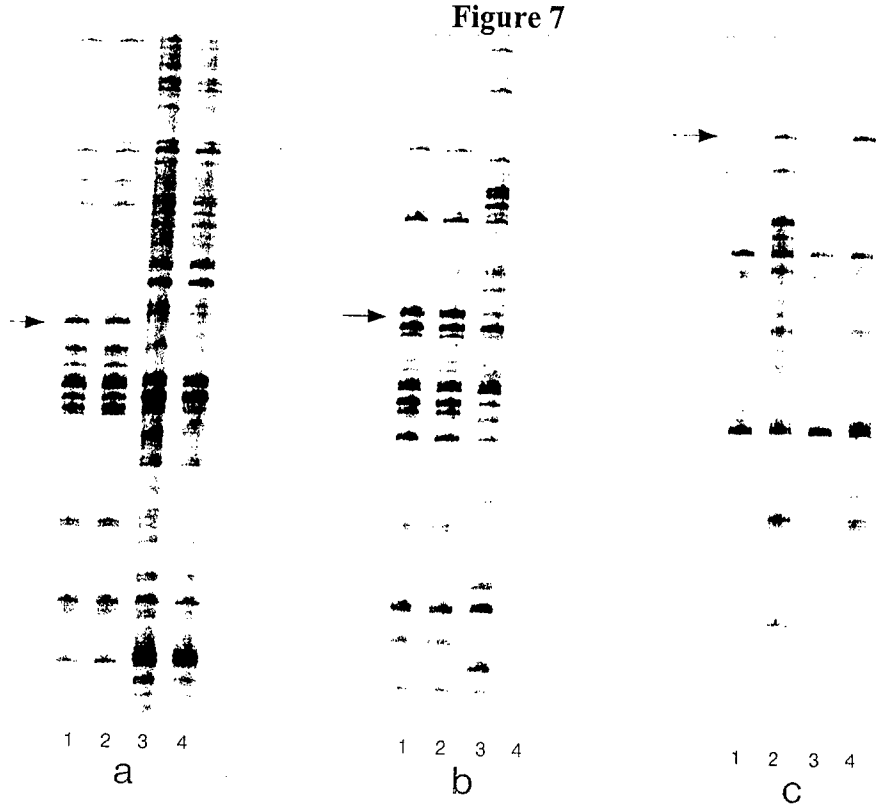

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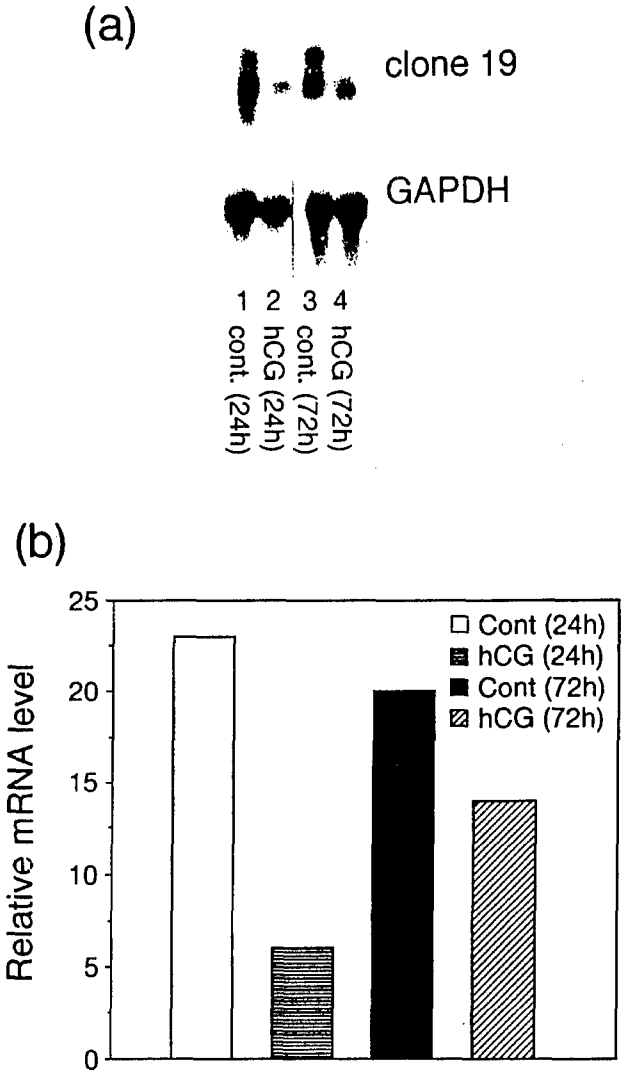
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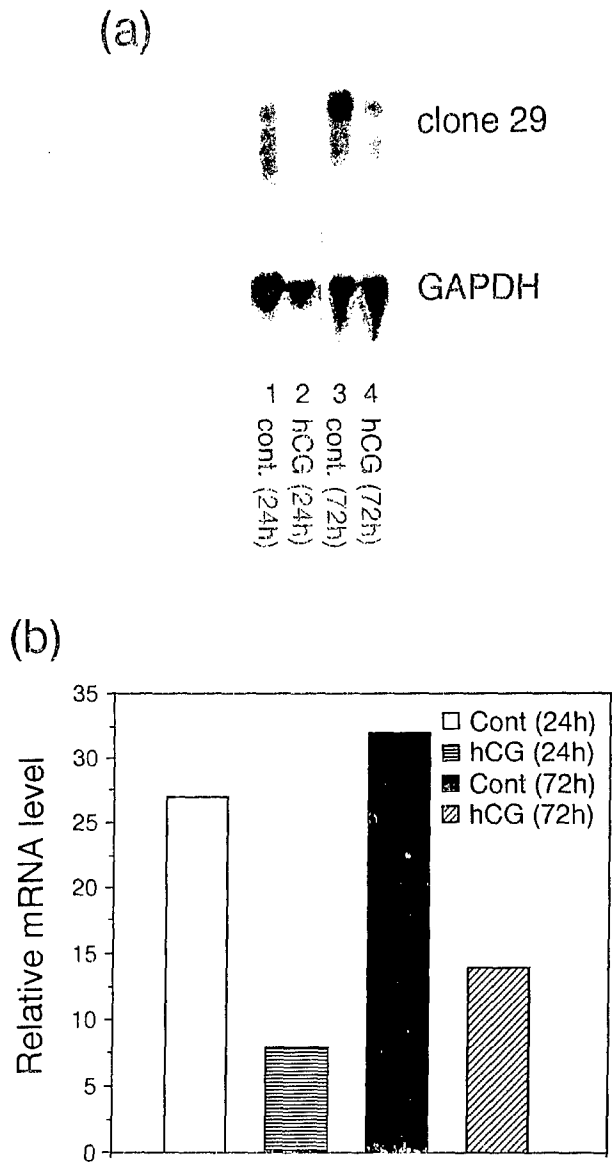
clone 19
GAPDH
1 cont. (24h)
2 hCG (24h)
3 cont. (72h)
4 hCG (72h)
(b)
Relative mRNA level
25
20
15
10
5
0
Cont (24h)
hCG (24h)
Cont (72h)
hCG (72h)

Figure 9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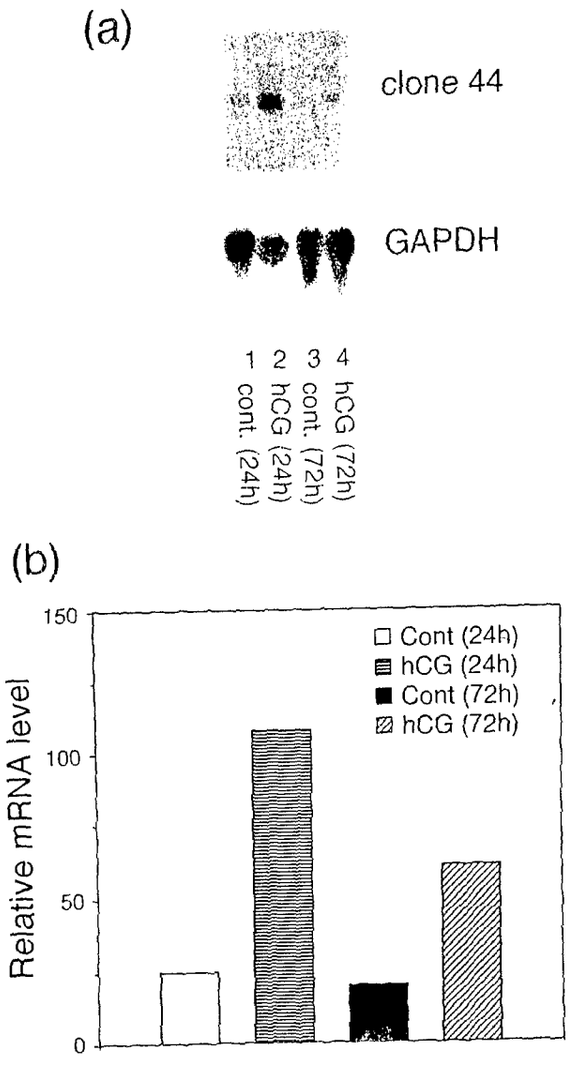
clone 29
GAPDH
1 cont. (24h)
2 hCG (24h)
3 cont. (72h)
4 hCG (72h)
(b)
Relative mRNA level
35
30
25
20
15
10
5
0
Cont (24h)
hCG (24h)
Cont (72h)
hCG (72h)

Figure 10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GAPDH
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2 hCG (24h)
3 cont. (72h)
4 hCG (72h)
(b)
Relative mRNA level
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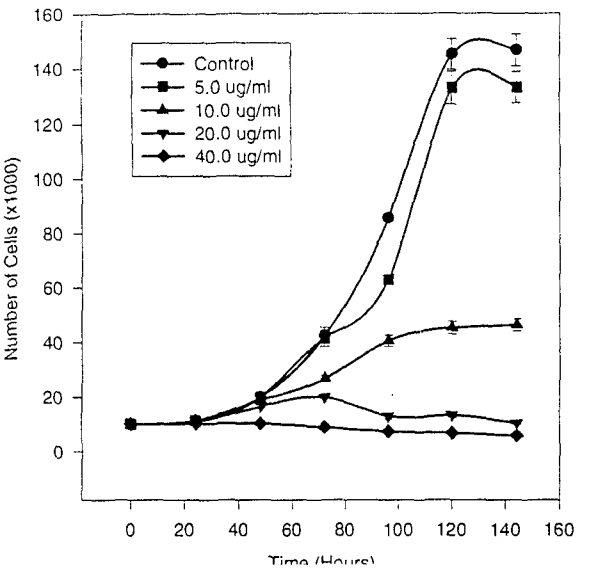
100
50
0
Cont (24h)
hCG (24h)
Cont (72h)
hCG (72h)

Figure 11



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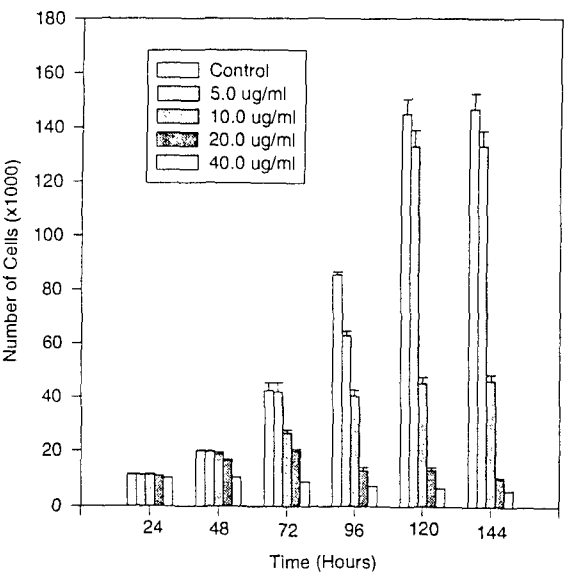

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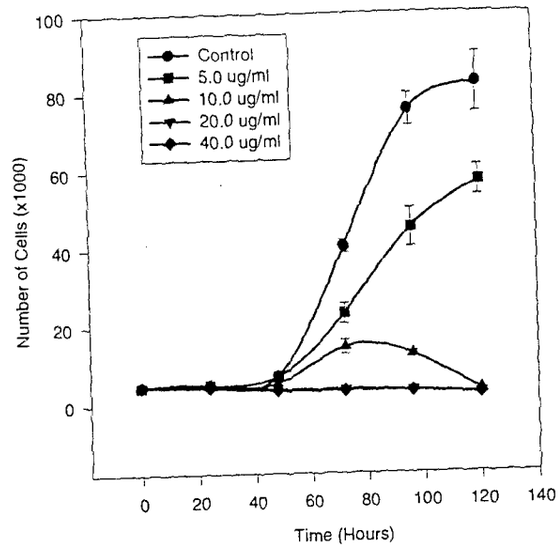

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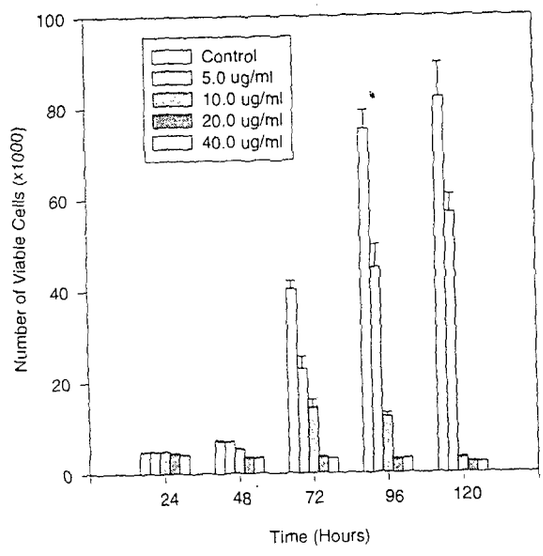

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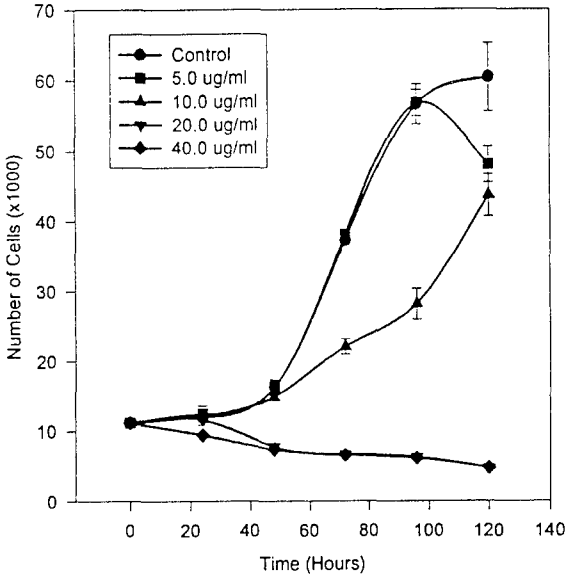

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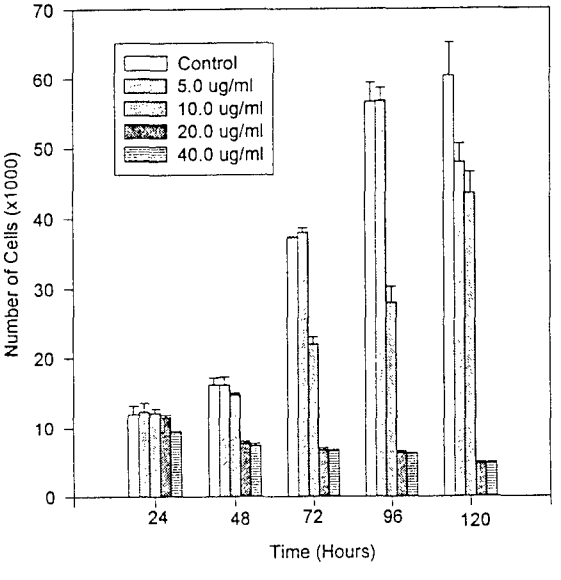

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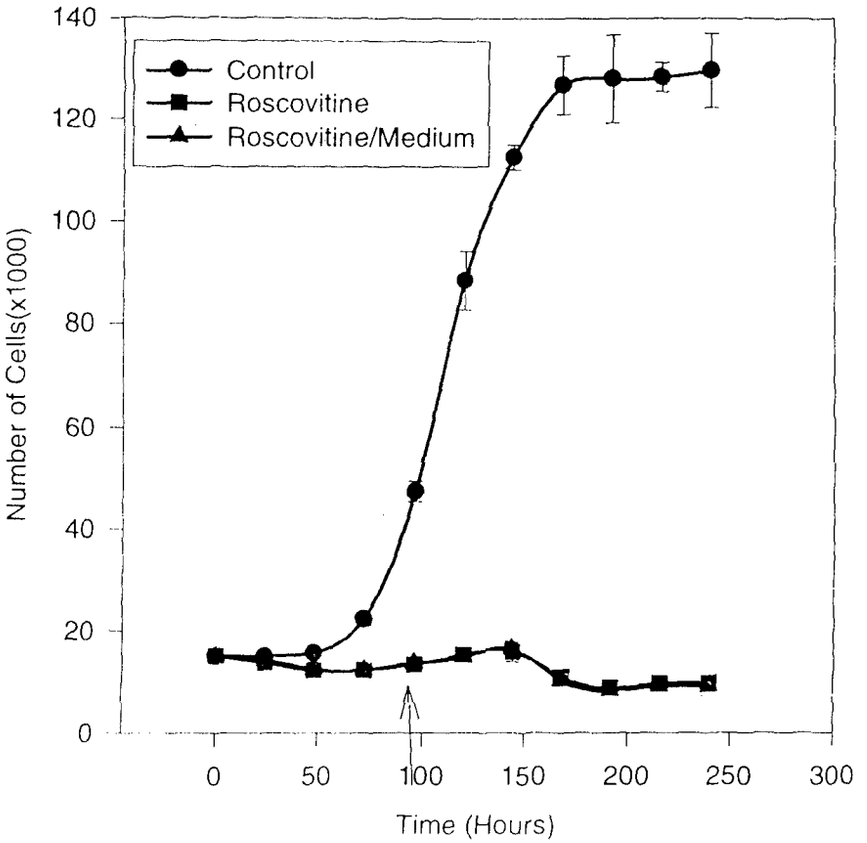

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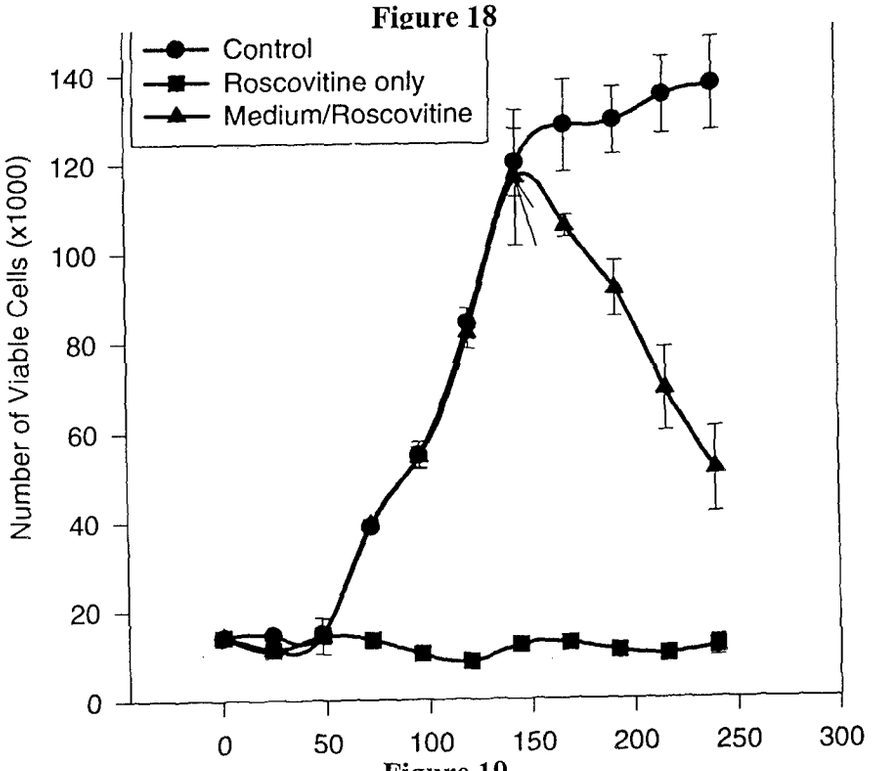

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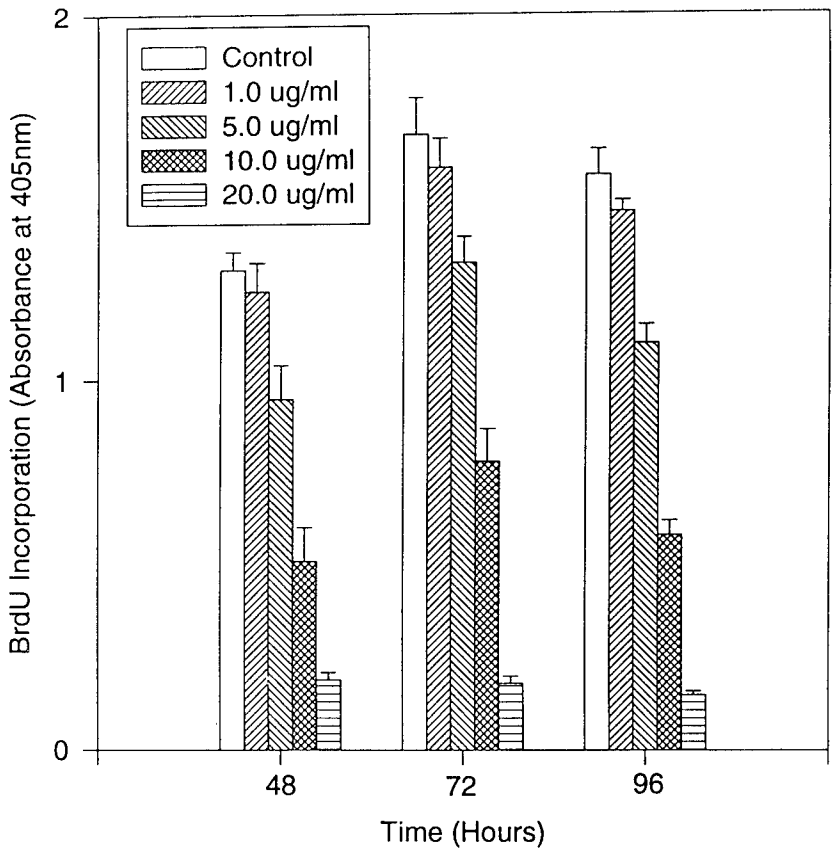

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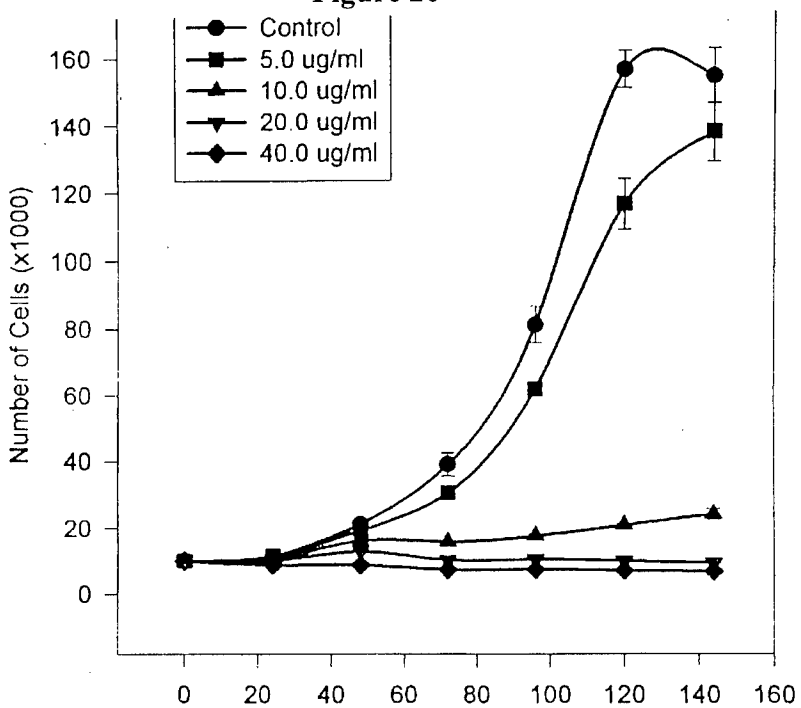

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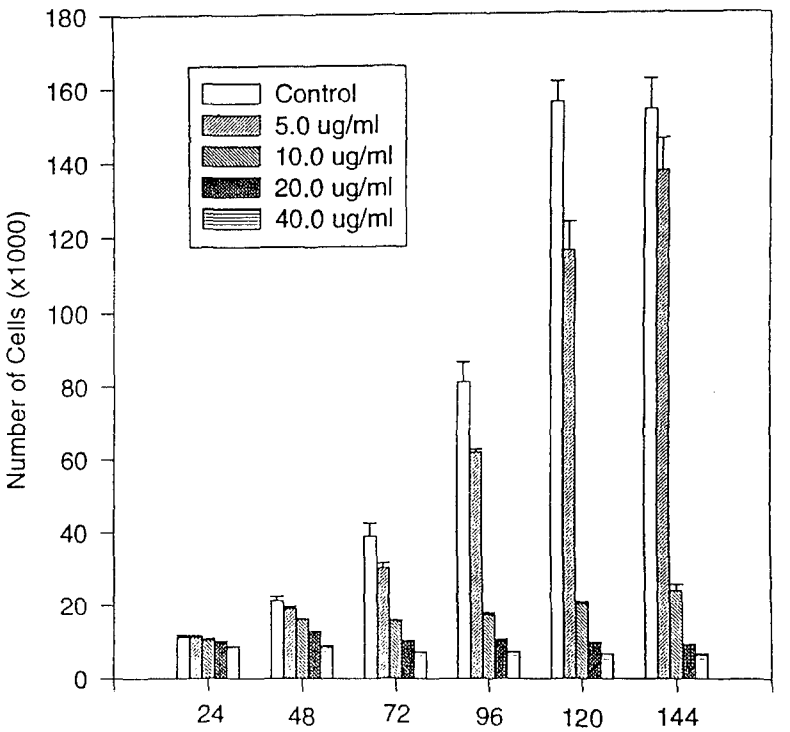

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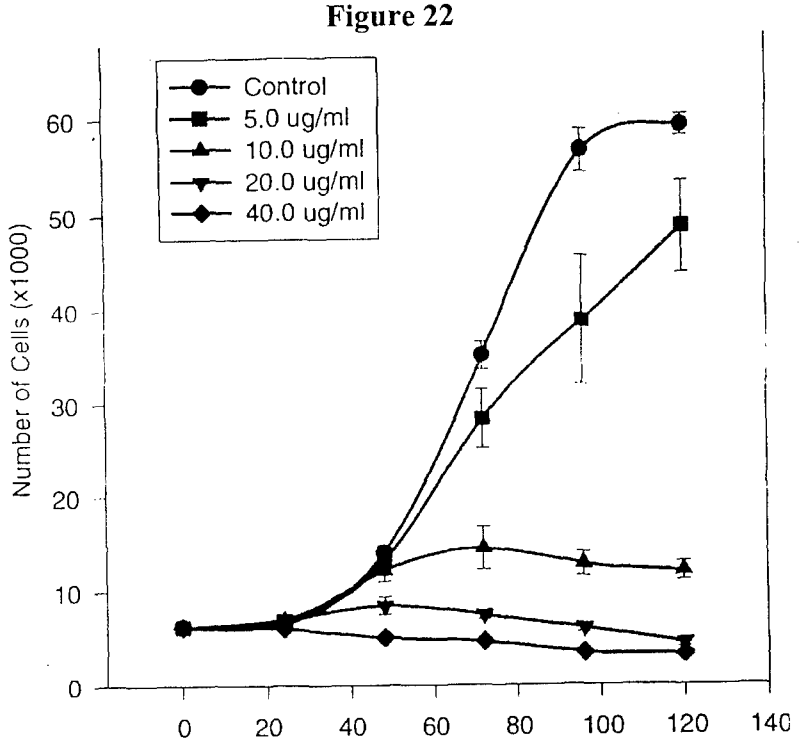

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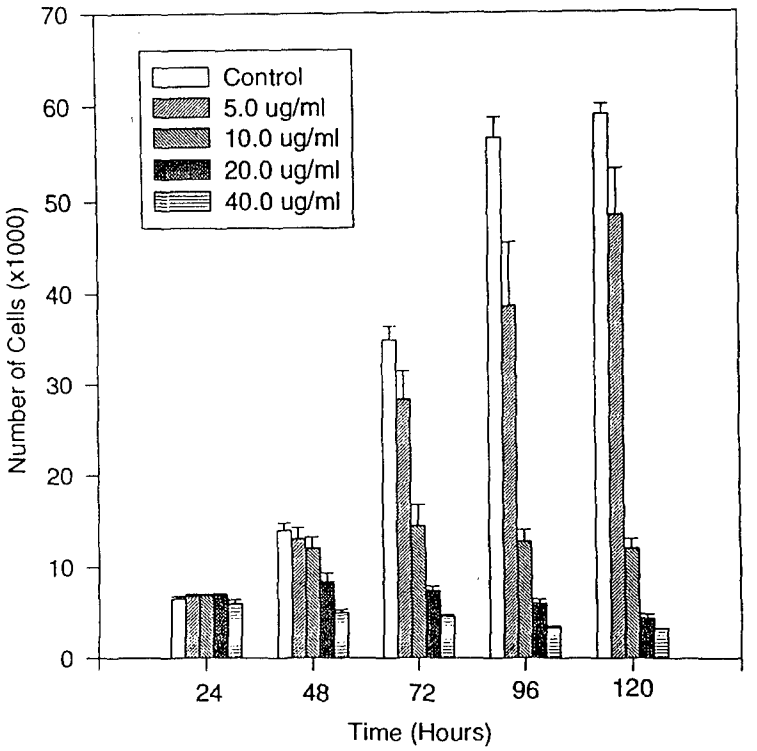

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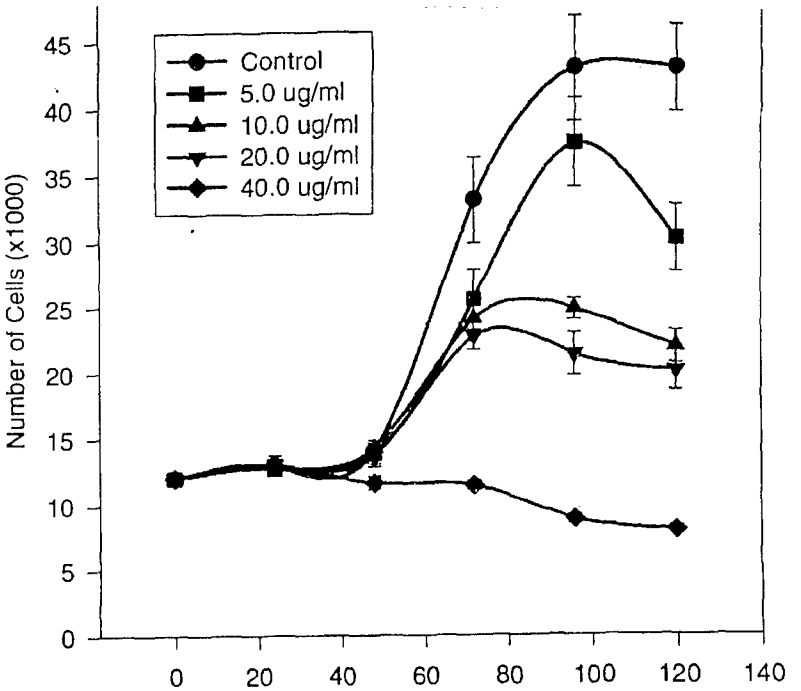

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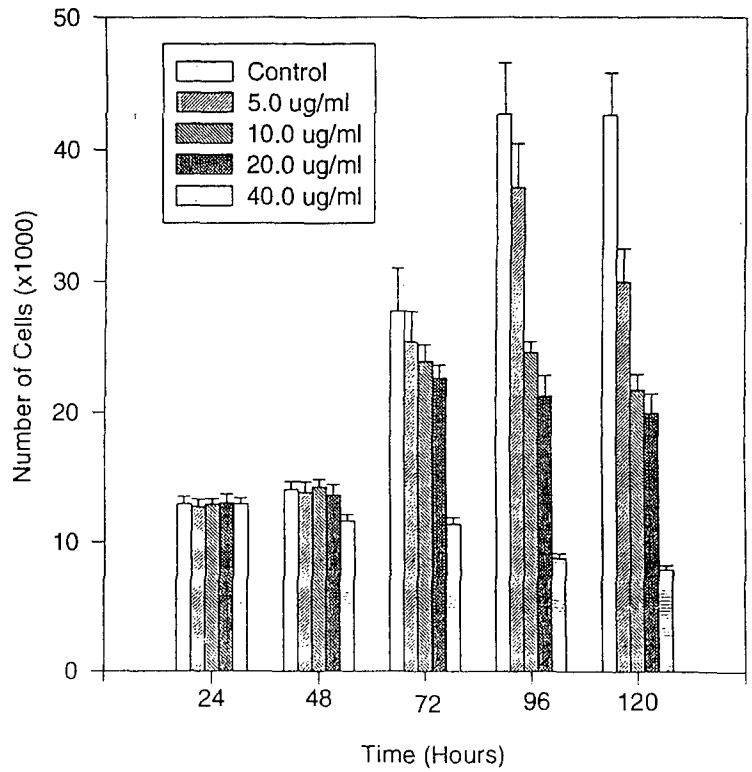
Control
5.0 ug/ml
10.0 ug/ml
20.0 ug/ml
40.0 ug/ml
Number of Cells (x1000)
0
10
20
30
40
50
24
48
72
96
120
Time (Hours)

Figure 26

LIST OF PUBLICATIONS

1. Russo, I.H. and Russo, J. Role of hormones in cancer initiation and progression. J. Mammary Gland Biol. & Neoplasia. 3:49-61,1998.

2. Mgbonyebi, O.P., Russo, J. and Russo, I.H. Antiproliferative effect of synthetic reverastrol on human breast epithelial cells. Int. J. of Oncol. 12:865-869, 1998.

3. Mgbonyebi, O.P., Russo, J. and Russo, I.H. Roscovitine inhibits the proliferative activity of immortal and neoplastic human breast epithelial cells. Anticancer Res.18:751-756, 1998..

4. Russo, J. and Russo, I.H. Human chorionic gonadotropin in breast cancer. In: Endocrine Oncology (Ethier. S. Ed.). Humana Press, 1998

5. Salicioni, A.M., Russo, I.H. and Russo, J. Correlation between cell cycle regulators and the immortalization and transformation of human breast epithelial cell lines. Int. J. of Oncology, 13: 65-71, 1998.

6. Srivastava, P., Silva, I.D.C.G.; Russo, J., Mgbonyebi, O. P.; and Russo, I.H. Identification of genes differentially expressed in breast carcinoma cells treated with chorionic gonadotropin. Int. J. of Oncology 13: 465-469, 1998.

7. Mgbonyebi, O.P., Russo, J. and Russo, I.H. Roscovitine induced cell death and morphological changes indicative of apoptosis in MDA-MB-231 breast cancer cells. Anticancer Research, (In Press) (1998).

8. Srivastava, P., Russo, J., Mgbonyebi, O.P.; and Russo, I.H. Human chorionic gonadotropin (hCG) activates programmed cell death gene expression in human breast epithelial cells. Anticancer Research, Submitted (1998).

ABSTRACTS PUBLISHED

1. Salicioni, A.M., Higgy, N.A., Russo, I.H and Russo, J. Differentially displayed genes induced by chorionic gonadotropin in human breast epithelial cells. Proc. Differential Display and Related Techniques for Gene Discovery, Cold Spring Harbor, New York, October,1996, p107

2. Mgbonyebi, O.P., Russo, J. and Russo, I.H. Induction of Reversible growth arrest of immortal and neoplastic human breast epithelial cells by human chorionic gonadotropin (hCG). Proc. Am. Assoc. Cancer Res. 38:1977a, 1997.

3. Russo, I.H., Srivastava, P., Mgbonyebi, O.P., and Russo, J. Activation of Programmed Cell Death by Human Chorionic Gonadotropin in Breast Cancer Therapy. Acta Haematologica, 98: S1,16, 1997.

4. Russo, I.H., and Russo, J.., Molecular basis of breast cancer prevention. Proc. Of the 15th Annual Symposium of the European Cancer prevention Organization. Premalignant Markers of Breast Cancer. Bruges, Belgium, June, 26-28, 1997.

5. Salicioni, A.M., Russo, J., and Russo, I.H. Effect of human chorionic gonadotropin (hCG) on different cell cycle regulators in human breast epithelial cells MCF-10F. Proc. Am. Assoc. Cancer Res., 39:73a, 1998.

6. Srivastava, P., Silva, I.D.C.G., Mgbonyebi, P., Russo, J., Russo, I.H. Identification of genes differentially expressed in human breast carcinoma cells treated with human chorionic gonadotropin.. Proc. Am. Assoc. Cancer Res., 39:777, 1998.

7. Higgy, N.A., Russo, J., Mgbonyebi, P., Salicioni, A.M. and Russo, I.H. Human chorionic gonadotropin inhibits telomerase activity in human breast epithelial cells *in vitro.* Proc. Am. Assoc. Cancer Res. 39:3678, 1998.

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